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MOLECULAR MECHANISMS REGULATING THE G2 CHECKPOINT INDUCED AFTER DNA DAMAGE

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Discipline of Life Sciences

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ABSTRACT

The checkpoint kinase (chk) 1 is an essential component of the DNA damage checkpoint acting as the effector of the DNA damage to block the cell cycle at the G2-M transition. The studies reported here were aimed at elucidating the molecular mechanisms regulating the G2 checkpoint after DNA damage and at investigating Chk1 as a potential therapeutic target for new cancer treatments. The promoter region of the human Chk1 gene was at first isolated and characterized. It was possible to show a p53 dependent downregulation of the Chk1 gene. The transcriptional factor E2F1 was also found to have a role in modulating Chk1 promoter activity. To further understand the interplay between p53 and Chk1 in the G2 checkpoint, the cellular response to anticancer treatments in isogenic cellular systems differing in the expression of Chk1 and p53 was studied. The expression of Chk1 was downregulated by siRNA, in the HCT-116 colon carcinoma cell line and in its isogenic systems lacking either p53 or p21. The inhibition of Chk1 in p21^{-/-} and p53^{-/-} cell lines caused a greater abrogation of the G2 block and a greater sensitization to anticancer treatments than in the parental cells with an intact G1 checkpoint. Tetracycline-inducible expressing Chk1 siRNA clones have been obtained in HCT-116 wt and p53 deficient cells and have been transplanted in nude mice. Experiments are undergoing to verify the downregulation of Chk1 upon addition of tetracycline in mice drinking water. The last part of my thesis focused on the investigation of the physiological role of Chk1 in cancer somatic cell lines. The understanding of this aspect appears particularly important as Chk1 has been proposed to be a potential drug target for anticancer therapy. The effects of the lack of Chk1 by siRNA transfection were studied in different human somatic cell lines. The

downregulation of Chk1 by siRNA in the HCT-116 isogenic cellular systems did not alter cell growth or compromise survival of these cells independently from the status of p53 and p21. On the other hand, in different cell lines, like U2OS and HeLa, an effect in cell growth, and in the cell phenotype was observed. These data suggest that the lack of Chk1 may have different consequences depending on the intrinsic genetic background of the cell line studied.

CHAPTER 1

INTRODUCTION

1.1 CELL CYCLE REGULATION, CHECKPOINTS AND CANCER

The cell cycle is a succession of very well organized molecular events that give the ability to the cell to produce the exact itself's copy. The DNA replication and the segregation of replicated chromosomes are the main events of the cell cycle. The DNA replication occurs during the so called S phase (synthetic phase) which is preceded by the DNA synthesis preparatory phase (Gap1 or G1 phase), whereas the nuclear division occurs in mitosis (M phase) and is preceded by the mitotic preparatory phase (gap 2 or G2 phase). The G1, S and G2 phases represent the interphase of a proliferating cell and constitute the time lapse between two consecutive mitoses. The differentiated cells that do not proliferate enter in the so called G0 phase which is a steady state phase or resting phase (Nurse, 1994; Nurse, 1994; O'Connell and Nurse, 1994; Vermeulen et al., 2003).

The progression of a cell through the cell cycle is strictly regulated by key regulatory proteins called CDK (cyclin dependent kinase) which avoid the initiation of a cell cycle phase before the completion of the preceding one (figure 1.1-1.2) (Sherr and Weber, 2000; Vermeulen et al., 2003). The cdks are a family of serine/threonine protein kinases that are activated at specific points of the cell cycle consisting of a catalytic subunit with a low intrinsic enzymatic activity and of a fundamental positive regulatory subunit called cyclin (Morgan, 1995; Lees, 1995; Arellano and Moreno, 1997; Pavletich, 1999). Cyclin protein levels rise and fall during the cell cycle, activating the corresponding cdk, whereas the cdk protein levels are kept constant throughout the cell cycle. Once the complex cdk-cyclin is formed, it gets activated by the protein CAK (cdk activating protein) which phosphorylates the complex ensuring the subsequent phosphorylation of target gene products required for the progression of the cell through the cell cycle

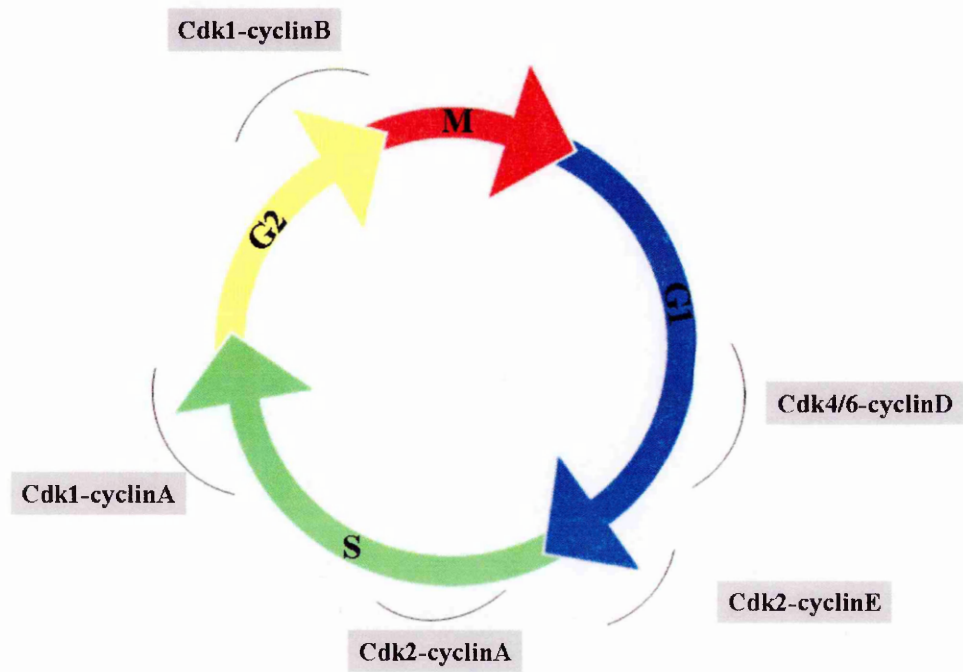


Figure 1.1 Cell cycle phases and main cell cycle regulators

A cell cycle scheme with the main cyclins and CDK regulators proteins is represented.

(Morgan, 1995; Pines, 1995a). When quiescent cells are stimulated by mitogen signals, CDK4 and CDK6 are activated by association with D type cyclins. These above cited cdk-cyclin complexes are important for the progression through the G1 phase and the restriction point preparing the cell to the replicative phase by phosphorylating the oncosuppressor protein pRb which causes the activation of the E2F family transcription factors. The activation of CDK4 and CDK6 is followed by the subsequent activation of CDK2 by cyclin E and cyclin A, which in turn initiates DNA replication. As the DNA replication process finishes, the Cdk1/cyclin B complex is activated leading to mitosis (Sherr, 2000; Vermeulen et al., 2003). The activation status of the cdk-cyclin complexes is also monitored by negative regulation of the ATP binding site by phosphorylation in specific residues and subsequent reactivation by specific phosphatases which dephosphorylate the same residues. Inhibitory proteins also contribute to negatively regulate the cdk_s by forming either binary complexes with cdk_s or ternary complexes with cyclin cdk dimers (figure 1.2). Three distinct families of these so called cyclin dependent kinase inhibitors (CKI) can be distinguished (Sherr and Roberts, 1999). The first one is called INK family and is composed by four members: p15, p16, p18 and p19. They mainly regulate the G1-S transition of the cell cycle targeting to CDK4 and CDK6 by binding the cdk subunit and causing a conformational change of the kinases which become inactive precluding the cyclin binding (Russo et al., 1998). The second family of inhibitors is the Cip/Kip family and consists of three members: p21^{cip1}, p27^{kip1} and p57^{kip2}. The components of this group negatively regulate the cdk2/cyclinA and cdk2/cyclinE complexes whereas they positively regulate the cdk4/6 cyclinD complexes by facilitating and stabilizing

the association of cyclin and CDKs (Cheng et al., 1999). The final class of inhibitors is the pRb protein family which consists of two members: p107 and p130. These proteins, better known as transcriptional inhibitors, act as potent cyclin E/A-cdk2 inhibitors by binding both to cyclin and to cdk sites (Cobrinik, 2005).

An additional level of cdk regulation is the control of nuclear import/export which can be easily exemplified by the cyclinB1-Cdk1 complex that is kept out of the nucleus through an active nuclear export until late G2, when the nuclear exporting signals are inactivated by phosphorylation ensuring nuclear accumulation. The regulation of the Cdk1-cyclinB1 complex *via* cytoplasmic sequestration together with the negative regulatory phosphorylation of Cdk1 prevents premature phosphorylation of mitotic targets and the entry in mitosis (Yang et al., 1998). Other examples are the CDK inactivating kinases Wee1 and Myt1 located respectively in the nucleus and Golgi complex protecting the cells from premature mitosis and the 14-3-3 group of proteins that regulate the intracellular trafficking of different proteins such as the phosphatase Cdc25C (Liu et al., 1997; Peng et al., 1997). The above mentioned events are very well monitored by signaling pathways called checkpoints which constantly make sure that upstream events are successfully completed before the initiation of the next phase. It's in fact important that alterations in duplication of the DNA during S phase do not occur, to avoid the segregation of aberrant genetic material to the daughter cells hence ensuring accurate genetic information's transmission throughout cellular generations. Lack of fidelity in cell reproduction processes creates a situation of genetic instability which contributes to the development of cancer disease. In cancer, the genetic control of cell division is altered resulting in a massive cell proliferation. Mutations mainly occur in

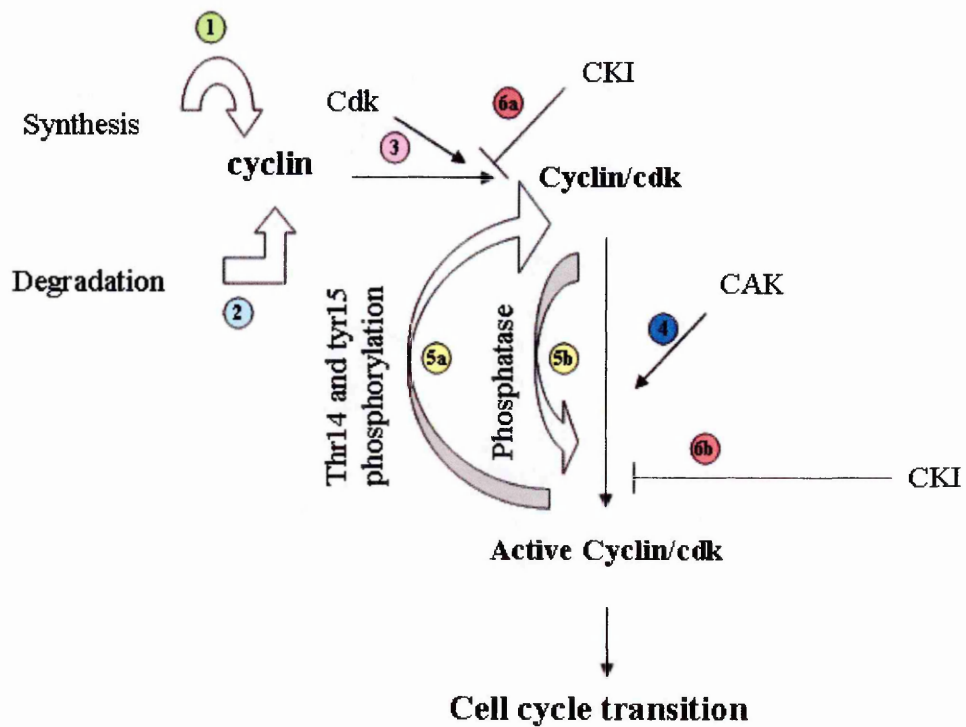


Figure 1.2 Schematic recapitulation of the levels of regulation of the cyclin dependent kinases (Cdk)

1 and 2. Synthesis and degradation of cyclins at specific stages of the cell cycle. 3. Association of cdk's to cyclins in order to be active. 4. Activation of the cdk/cyclin complexes by CAK. 5. Inactivation of cdk/cyclin complexes by phosphorylation at thr14 and tyr15 (5a) and reactivation by phosphatases acting on these sites (5b). 6. Cdk inhibitor proteins (CKI) preventing either the assembly of cdk/cyclin complexes (6a) or the activation of the cdk in the complex (6b). The activated cdk/cyclin complexes can phosphorylate substrates necessary for transition to the next cell cycle phase.

two classes of genes: proto-oncogenes and tumor suppressor genes. In normal cells the proto oncogenes products act at different levels in pathways that stimulate proper cell proliferation while the mutated proto-oncogenes or oncogenes can promote tumor growth due to uncontrolled cell proliferation. Tumor-suppressor genes normally keep cell numbers down, either by halting the cell cycle and thereby preventing cellular division or by promoting programmed cell death. When these genes are rendered non-functional through mutation, the cell becomes malignant. Defective proto-oncogenes and tumor-suppressor genes act similarly at a physiologic level: they promote the inception of cancer by increasing tumor cell number through the stimulation of cell division or the inhibition of cell death or cell cycle arrest. Uncontrolled cell proliferation which evolves in cancer can occur through mutation of proteins important at different levels of the cell cycle such as CDK, cyclins, CKI and CDK substrates. Defects in cell cycle checkpoints can also result in gene mutations, chromosome damages and aneuploidy all of which can contribute to tumorigenesis (Sherr, 1996; McDonald, III and El Deiry, 2000).

Understanding the molecular mechanisms regulating the cell cycle progression and how these processes are altered in malignant cells may be crucial to better define the events behind such a complex and devastating disease like cancer.

1.2 DNA DAMAGE CHECKPOINT

A faithful transmission of genetic informations from one cell to its daughters requires the ability of a cell to survive to spontaneous and induced DNA damage to minimize the number of heritable mutations. To achieve this fidelity, cells have evolved surveillance mechanisms composed by an intricate network of checkpoint proteins that tells the cell

to stop or delay the cell cycle progression providing enough time for DNA repair. When the damage can not be repaired cells undergo apoptosis.

Many different lesions can occur in the cells which are coupled to different repair mechanisms. First, normal metabolic processes or exposure to external ionizing radiations generate free oxygen radicals and can break the phospho diester bonds in the backbone of the DNA helix (single strand break). When two of these breaks are close to each other but on opposite DNA strands, a double strand break (DSB) is present. Second, alkylating agents can modify purine bases and can cause intra strand or inter strand crosslinks. Inhibitors of DNA topoisomerase can cause DNA lesions leading to enhanced single or double strand break depending on which topoisomerase is inhibited and on the phase of the cell cycle. Different mechanisms are required to repair the damage to the DNA backbone or to the DNA bases and the repairing mechanisms may also vary depending on the different phases of the cell cycle (Kastan and Bartek, 2004; Froelich-Ammon and Osherooff, 1995). Briefly, the signal transduction pathway activated after DNA damage consists of many different components departing from sensor proteins that recognize the damage on the DNA and transmit the signals that are amplified and propagated by adaptors/mediators to the downstream effectors that connect the checkpoint with the cell cycle machinery (figure 1.3).

This molecular cascade is very well conserved from yeast to humans and nowadays the proteins involved in this pathway have been identified (Kastan and Bartek, 2004; Zhou and Elledge, 2000; Lukas et al., 2004b).

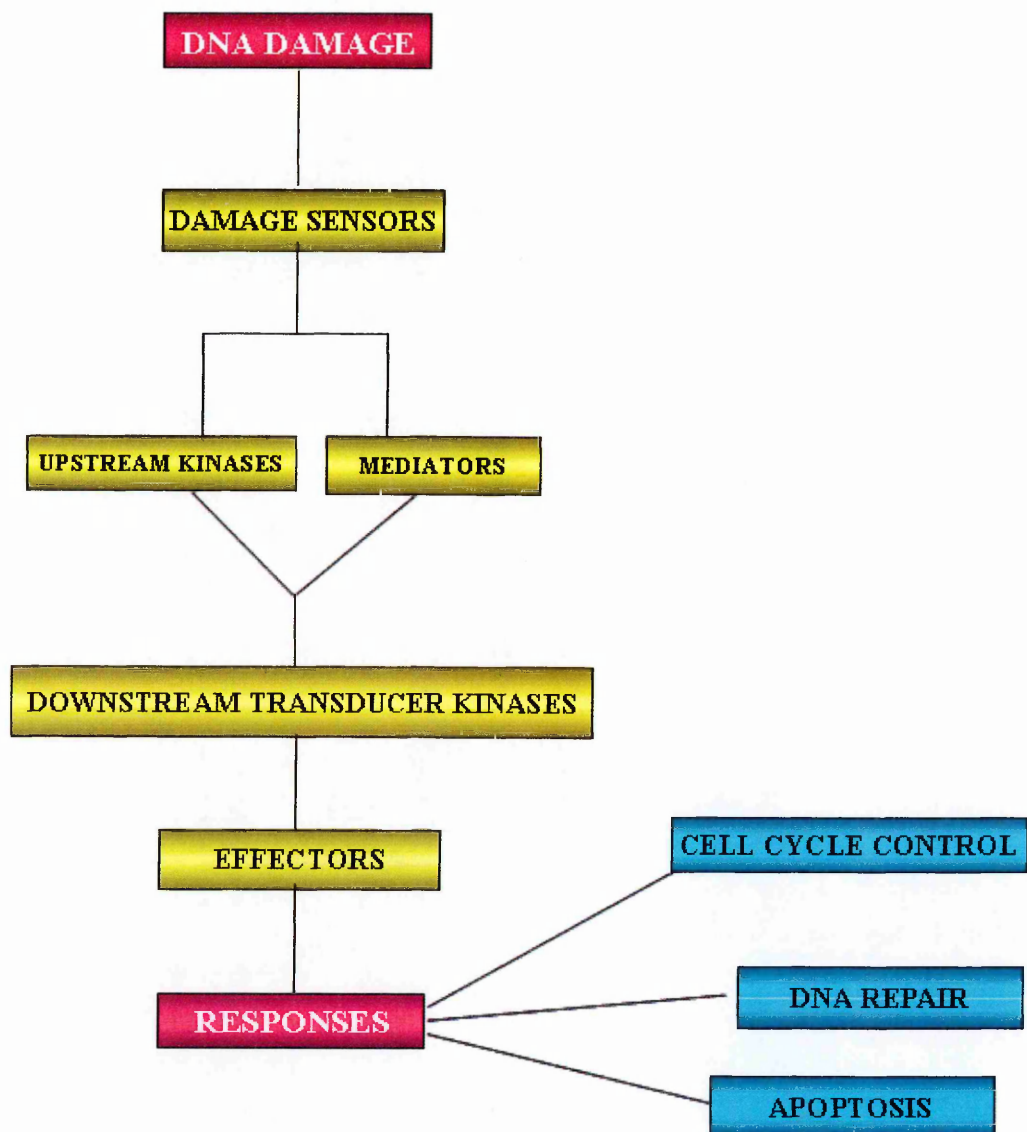


Figure 1.3 Schematic view of the general DNA damage response signal transduction pathway

1.2.1 GENERAL ORGANIZATION OF CHECKPOINTS COMPONENTS

After DNA damage exposure an arrest of the cell cycle progression should take place rapidly. This is ensured by the PI(3)K (phosphatidylinositol-3-OH kinase) - like kinase (PIKKs), ATM (ataxia telangiectasia mutated) and ATR (ATM-and Rad3 related), that are almost instantly activated following stress exposure and recognize the damage, initiating the DNA damage response (Durocher and Jackson, 2001; Lavin, 1999). They are both able to be quickly recruited to sites of DNA damaged where they form complexes with other proteins such as the replication factor RFC and PCNA (Shiloh, 2001; Balajee and Geard, 2001; Unsal-Kacmaz et al., 2002).

While the ATM kinase is activated following DNA damage, the ATR kinase is critical for cellular response to the arrest of the DNA replication forks. They are both large protein kinases that phosphorylate many substrates to achieve their physiological functions.

ATM is autophosphorylated in ser 1,981 after DNA DSB (double strand breaks) leading to a conformational change, causing the dissociation of a homodimer to monomer which can subsequently phosphorylate, at the nucleoplasmic level, its substrates like p53, NBS1 (Nijmegen breakage syndrome 1), BRCA1 (breast cancer 1) and SMC 1 (structural maintenance of chromosomes 1) at the sites of DNA breaks. The heterodimeric ATM does not need to bind to the DNA break to cause its autophosphorylation as it has been shown to sense changes in chromatin structure at some distance away from the site of the DNA break (Bakkenist and Kastan, 2003). It has been recently observed that the multiprotein complex MRE11 (meiotic recombination 11)/rad50/NBS1 (MRN) contributes to the activation of ATM after DSB (Uziel et al.,

2003; Horejsi et al., 2004). Patients, mice and cells lacking ATM are viable, suggesting that the ATM kinase is not essential for critical cellular functions such as normal cycle progression or cellular differentiation (Shiloh and Kastan, 2001).

ATR together with ATM is the most well characterized damage “sensor” (Abraham, 2001). It exists in a complex with the ATR interacting protein (ATRIP), both before and after exposure of stresses such as ultraviolet radiation. It has been observed that the *in vitro* binding of ATRIP to single stranded DNA is ensured by the replication protein A (RPA), a single stranded DNA binding protein involved in DNA replication. The binding of the ATRIP to RPA is determinant for ATR to localize to sites of replication fork arrest as the accumulation of RPA on ssDNA (single strand DNA) leads to the recruitment of the ATRIP protein and its heterodimeric partner ATR, which once localizes to the ssDNA regions can phosphorylate its substrates such as rad17 and Chk1 (Cortez et al., 2001; Zou and Elledge, 2003). In order for ATR to carry out its cellular functions, several other proteins and complexes have to be recruited to the ssDNA site, such as the clamp loading, Rad 17-containing complex, which is necessary for the loading of the RAD9-RAD1-Hus1 (9-1-1) sliding clamp into the chromatin and the claspin protein, which is independently recruited to chromatin as well (Osborn et al., 2002; Lin et al., 2004). Cells and animals lacking ATR are not viable thus suggesting that this protein has a critical role in the normal cell cycle progression independently on the presence of cellular stress. Recent evidences attributed to ATR a crucial role in the normal progression of the DNA replication forks and consequently in all cellular responses that share inhibition of replication fork progression as in the presence of DNA damage, whereas ATM seems to be activated just following DSB (Brown and

Baltimore, 2003; Shechter et al., 2004). The activity of ATM and ATR is modulated by the checkpoint mediators. They act facilitating the interactions between ATM/ATR and their substrates, helping to provide signal transduction specificity and mediating the spatio temporal assembly of multiproteins complexes in the chromatin regions surrounding the DNA damage sites. As they are recruited to the sites of DNA damage independently of ATM and ATR, they could have the ability to sense the DNA lesions as well (Goldberg et al., 2003). The ATM related mediators include MDC1, 53BP1 and BRCA1. These are large multi domain proteins containing 2 tandem BRCT (BRCA-1 carboxy terminal) domains at their C terminus working promoting protein-phosphoprotein binding modules to facilitate eventually the transient multiple interactions of checkpoints and repair proteins near the DNA damage sites (Stewart et al., 2003; Manke et al., 2003; Wang et al., 2002). Their accumulation to sites of DNA damage depends on ATM mediated phosphorylation of histone H2AX. MDC1 represents one of the earliest proteins to localize at DNA DSB and it works like a molecular bridge between the γ H2AX and the NBS1 component of the MRN complex helping to facilitate interactions for these and other checkpoints and the repair proteins (including the activated ATM) in the proximity of the DNA damaged sites (Celeste et al., 2003; Lukas et al., 2004a; D'Amours and Jackson, 2002).

On the other hand, the ATR controlled checkpoint signaling, through Chk1 activation, relies on a different mediator called claspin which is structurally unrelated to the mediators involved in DSB. It interacts with chromatin structures created by active replication forks, ensuring ATR mediated phosphorylation events being so related to Chk1 activation (Chini and Chen, 2004; Lee et al., 2003).

In general the multiprotein interactions mediated by these mediators contribute to ensure a prompt checkpoint response. In fact cells lacking any of these mediators show enhanced sensitivity to DNA damaging agents such as IR (ionizing radiation) and impaired intra S-phase and G2/M cell cycle checkpoints.

As I already mentioned, the ATR and ATM mediated phosphorylations trigger respectively and preferentially the activation of the so called checkpoint downstream kinases Chk1 and Chk2. The ATM-Chk2 and ATR-Chk1 cascades modules share many substrates among the checkpoint effector proteins which are for example the three human phosphotyrosin phosphatases, Cdc25A, B and C that dephosphorylate the cyclin dependent kinases that act on proteins directly involved in cell cycle transitions (Zhou and Elledge, 2000; Bartek and Lukas, 2003).

1.3 G1 CHECKPOINT

To prevent the entry in S phase in the presence of DNA damage, the G1 checkpoint is activated in the cells thus inhibiting the initiation of DNA replication. Under normal conditions cells gain the ability to enter the S phase at a stage called restriction point which in mammalian cells precedes the DNA synthesis by about two hours, but when DNA damage is introduced, the DNA synthesis is prevented even if the cells have reached the restriction point. In the G1 checkpoint response, two distinct effectors, the Cdc25A phosphatase and the p53 transcription factor are phosphorylated and activated by the transducing kinases ATM/ATR and Chk1/Chk2 and more specifically depending on the kind of damage either by the ATM/Chk2 or by the ATR/Chk1 pathway. The phosphorylation of these two effectors occurs rapidly and simultaneously although the

impact of these events on cell cycle machinery is faster in case of the Cdc25A cascade not requiring transcription and accumulation of new synthesized proteins like in the p53 pathway (Bartek and Lukas, 2001). The phosphorylation of Cdc25A on serine residues by Chk1 and Chk2 kinases results in its inactivation by nuclear exclusion and ubiquitin-mediated proteolytic degradation (Sorensen et al., 2003; Zhao et al., 2002). Once inactivated, the Cdc25A can not anymore induce the activatory dephosphorylation of the catalytic subunit of cyclinE/Cdk2 and cyclinA/cdk2, thus preventing the initiation of the DNA synthesis by inhibiting the loading onto chromatin of Cdc45, a protein required for recruitment of DNA polymerase α into the assembled pre-replication complexes (Mailand et al., 2000; Falck et al., 2001). The checkpoint pathway that targets Cdc25A is implemented rapidly, independently on a transcriptional event and it delays the G1-S transition only for few hours (Molinari et al., 2000). On the other hand the activation of the p53 dependent mechanism ensures the maintenance of the G1 arrest in response to DNA damage (Figure 1.4) (Bartek and Lukas, 2001). This protein is present at low levels in unstressed conditions but after damage a very rapid accumulation in the nucleus can be observed because of the detachment of its binding protein mdm2 as a consequence of translational modifications, especially N terminal phosphorylations in the region which binds mdm2. In addition mdm2 is also targeted after DNA damage by ATM/ATR (Khosravi et al., 1999; Maya et al., 2001). The p53 stabilization and activation mediates the maintenance of the G1/S checkpoint arrest, which becomes fully operational several hours after the detection of DNA damage (Bartek and Lukas, 2001). After DNA damage p53 gets phosphorylated by the protein kinases Chk1 and Chk2 and also directly by the upstream checkpoint kinases ATM and ATR particularly on ser15

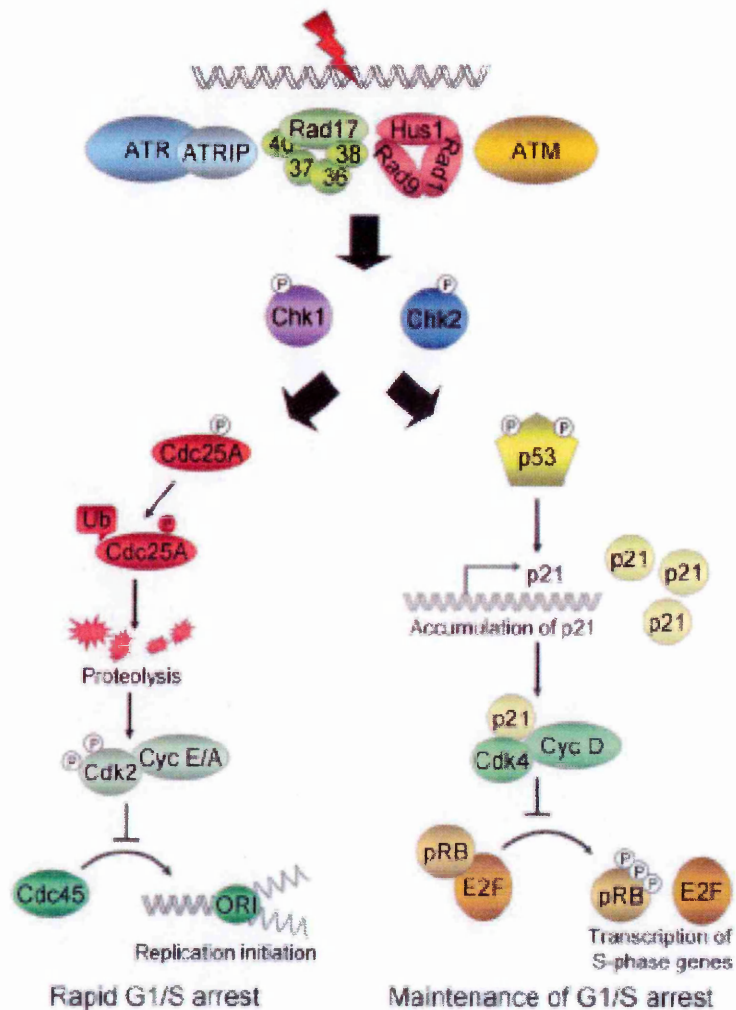


Figure 1.4 G1 checkpoint: molecular players

Schematic representation of the main molecular mechanisms involved in the activation of the G1 checkpoint after DNA damage. (Modified by Sancar A. et al. 2004).

(Shiloh, 2003; Kastan and Lim, 2000). Once activated, p53 transcriptionally transactivates its target genes, including p21^{WAF-1/Cip1} which binds to and inhibits the S-phase promoting Cdk2-CyclinE complex, thus maintaining the G1/S arrest. p21^{WAF-1/Cip1} also binds to the Cdk-CyclinD complex and prevents it from phosphorylating Rb, which once phosphorylated releases the E2F transcription factor required for the transcription of S-phase genes in order for S phase to proceed (Bartek and Lukas, 2001; Harper et al., 1993; Lin et al., 2001).

Although there is an evidence that p21 transcription can be induced following DNA damage even in the absence of p53 (Vikhanskaya et al., 1995), its transcription is mostly controlled by p53, and cells lacking the DNA binding domain of p53 or presenting mutations in this domain, fail to activate p21 and have a strongly reduced ability to induce G1 arrest (Fan et al., 1995; Wahl et al., 1996). The importance of p21 in mediating p53-induced G1 arrest has been further demonstrated by the generation of somatic p21^{-/-} cells which lack the ability to induce G1 arrest following damage induction, even in the presence of a functional p53 (Waldman et al., 1997).

1.3.1 THE ONCOSUPPRESSOR p53

A high percentage of human cancers present mutations in the p53 tumor suppressor protein as a result either of missense point mutations in the p53 gene or functional inactivation of the p53 protein by rapid degradation or by viral oncoproteins. In normal conditions p53 is present as a latent transcription factor with a short half life. DNA damage or oncogenic activation leads to the translational modification and stabilization of the p53 protein.

1.3.1.1 P53 GENE AND PROTEIN AND ITS REGULATION

The p53 gene, mapping on chromosome 17, codes for a protein of 393 aminoacids with an apparent molecular weight of 53 KDa. The cDNA coding for p53 is highly conserved among different species and it consists of different domains each characterized by a specific function (figure 1.5)(Haffner and Oren, 1995).

The central DNA binding domain confers to p53 the ability to act as a transcription factor and is particularly conserved through the evolution. The specific DNA sequence targeted by p53 is 5'PuPuPuCT/AT/AGPyPyPy3'(Levine, 1997). The N-terminus region constitutes a transcriptional activation domain which is able to interact with the basal transcription machinery and positively regulates gene expression. It is also the region recognized by the negative regulator of p53, mdm2 (Bottger et al., 1997). The crucial amino acids responsible for the binding of p53 to mdm2 have been identified thanks to the availability of the crystal structure of the N-terminal region of mdm2 with a p53-derived peptide containing amino acids 13-29 (Kussie et al., 1996). Interestingly this region of the protein contains different phosphorylatable aminoacidic residues that ensure the detachment of p53 itself from mdm2. It has been shown that the residues in p53 protein important for the interaction with mdm2 are mostly ser15 and ser20 which upon damage can be phosphorylated *in vitro* by different kinases, including ATM,ATR, DNA-PK, Chk1 and Chk2, depending on the kind of damage the cell has received (Meek, 1998; Kapoor and Lozano, 1998; Banin et al., 1998; Chehab et al., 2000; Shieh et al., 2000). Ser15 of p53 for example is clearly phosphorylated by ATM following IR and by ATR following UV (Meek, 1998; Kapoor and Lozano, 1998; Lane, 1998).

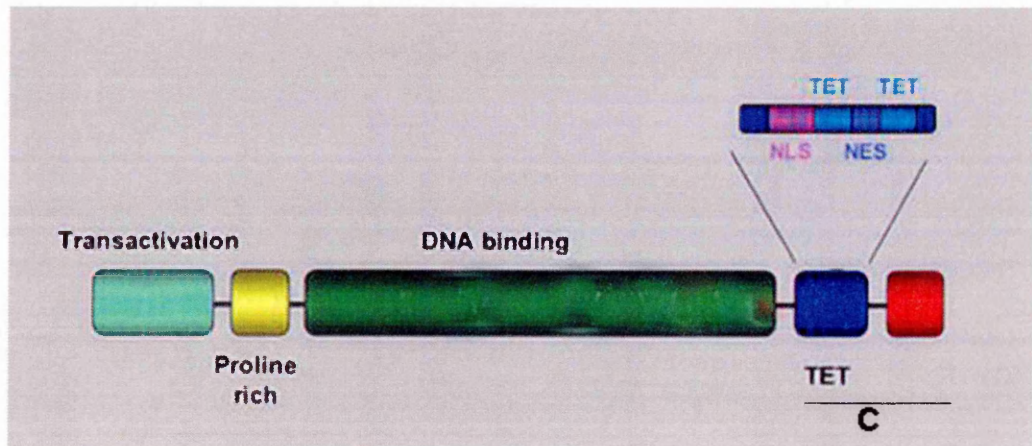


Figure 1.5 Structure and domains of the p53 protein

The amino terminal part comprises the acidic transactivation domain and the mdm2 protein binding site, following a series of conserved repeated proline residues. The central region contains the DNA binding domain which is the target of 90% of p53 mutations found in human cancers. The carboxy terminus of p53 contains the oligomerization domain necessary for p53 dimerization and the nuclear localization signals.

The C-terminal region contains the residues important for oligomerization, as the native p53 protein is present as a tetramer (Haffner and Oren, 1995; Oren, 1985). This region contains another DNA binding domain that recognizes the DNA in a structure specific manner rather than in a sequence specific way (Ahn and Prives, 2001). The p53 molecule is present in two conformationally distinct forms, one latent and the other active in sequence specific binding to DNA. One of the functions of the C-terminus of p53 is thought to be the ability to drive the molecule toward these two forms (Ahn and Prives, 2001; Hupp and Lane, 1994).

In normal, unstressed conditions, levels of p53 are very low. Immediately after a stress, p53 is induced by rising its levels rapidly and it accumulates in the nucleus (Vogelstein and Kinzler, 1992; Ko and Prives, 1996; Oren and Prives, 1996). This mechanism is mainly post-translational, and one of the crucial steps is mediated by the interaction of p53 with its regulator mdm2. In normal conditions, p53 is bound by mdm2, and this binding is a signal leading to ubiquitination and nuclear export (Michael and Oren, 2002; Haupt et al., 1997). Once ubiquitinated, p53 is degraded through the proteasome. Mdm2 exerts an E3 ubiquitin ligase activity towards p53 (Honda et al., 1997). The role of mdm2, however, is not only that of simply adding ubiquitin molecules to p53, but recent evidence indicates that there are consecutive regulated steps that occur before degradation of p53 takes place. In fact p53 ubiquitination occurs mainly in the nucleus, and p53, thus modified, is then exported through an active mechanism controlled by mdm2 into the cytoplasm, where it is degraded by cytoplasmic proteasome (Geyer et al., 2000; Boyd et al., 2000). To support this mechanism, mdm2 contains in its structure nuclear localization and

export signals which are necessary for its shuttling between nucleus and cytoplasm (Roth et al., 1998). The importance of mdm2 in the homeostasis of p53 has become clear when attempts to generate knock out mice for mdm2 failed due to embryo lethality. This lethality was shown to be avoided by preparing mdm2 knock out mice in a p53 null background (Montes de Oca et al., 1995). The reason why mdm2 mice did not proceed to birth is probably because levels of p53 are constantly high, lacking its main negative regulator, thus inducing growth arrest and/or apoptosis in all cells in the absence of stress induction (de Rozières et al., 2000).

The activation of p53 following damage needs the detachment of p53 itself from mdm2. Two distinct mechanisms have been described. The first one is, as I just mentioned, related to the ability of p53 to undergo phosphorylation at the N-terminus following stress induction. Another mechanism which mediates the detachment of p53 from mdm2 has been reported in cells activated by oncogenic transformation. In this case the mechanism leading to dissociation of mdm2 from p53 involves the p14^{arf} protein (Eischen et al., 1999). p14^{arf} is able to bind mdm2 at residues different from those necessary for the interaction with p53. Once bound by p14^{arf}, mdm2 is localized to nucleolar structures and sequestered (Pomerantz et al., 1998; Weber et al., 1999), thus resulting in a release of p53 and a rise in its levels.

Besides the region important for binding to mdm2, p53 is phosphorylated at other N- and C-terminal sites (Siliciano et al., 1997; Giaccia and Kastan, 1998; Meek, 1998). Different kinases responsible for these post-translational modifications have been identified, including Casein Kinase II (CKII), protein kinase C, CDKs (Giaccia and Kastan, 1998; Meek, 1998). These modifications are thought to be important for the

stabilization of sequence specific for DNA binding and possibly for determining the substrate specificity in transcriptional activation of downstream genes (Oren and Prives, 1996). For example phosphorylation at ser46 has been shown to be essential for the activation of apoptosis related genes (Oda et al., 2000b).

The acetylation is another post-translational modification of p53. It has been shown in fact that p53 is a substrate of HAT, being efficiently acetylated at the C terminus (Gu and Roeder, 1997). These acetylations, occurring at lysine 320, 372, 373, 382 and 381, mediated by the HATs P300 and pCAF were found in cells in response to various stimuli (Prives and Manley, 2001; Liu et al., 1999). The acetylation of p53 does not seem to stimulate the sequence specific binding, but could be important for the recruitment of transcriptional co-activators or for p53 localization (Prives and Manley, 2001), although this issue is yet to be defined.

1.3.1.2 P53 FUNCTION

P53 exerts its activity mainly as a transcription factor. Following activation, it activates the transcription of distinct classes of genes. Two important classes of genes are activated by p53: those involved in cell cycle arrest and in apoptosis (Waldman et al., 1997; Ko and Prives, 1996; Bates and Vousden, 1996; Vogelstein et al., 2000) depending on the kind and extent of damage. Two genes, p21 and bax, are among the most studied p53 downstream genes responsible for p53 induction of cell cycle arrest and apoptosis, respectively (El Deiry et al., 1993; Miyashita et al., 1994). Many other genes, however have been implicated in p53-mediated cell cycle arrest or apoptosis, including the 14-3-3 sigma protein, GADD45, FAS, Noxa, PUMA, the family of PIGs,

AIP (Vogelstein et al., 2000; Kastan et al., 1992; Hermeking et al., 1997; Muller et al., 1998; Oda et al., 2000a; Oda et al., 2000b; Yu et al., 2001; Nakano and Vousden, 2001). It has been shown that the critical decision between activating either apoptotic or growth arrest inducing genes is mediated both by the sequence of the DNA element and by the different post translational modifications of p53. The first evidence is dictated by the difference of the p53 responsive elements present in the DNA regulatory sequences of p21 and bax genes. The element present in the bax promoter, in particular, is less efficiently bound by p53 and, in transcription experiments using heterologous reporter genes, it has a weaker transcriptional response compared to the analogous element in p21 (De Feudis et al., 2000).

As already outlined the p53 post translational modifications are important as demonstrated by the observation that one of the p53 responsive genes involved in apoptosis, the AIP gene, is not efficiently transcribed, if p53 is not phosphorylated at residue serine 46, which probably induces a conformational change in p53 allowing efficient binding and transactivation ability for this specific gene (Oda et al., 2000b; Hofmann et al., 2002).

p53 can also exert its activity *via* transcriptional repression (Vogelstein et al., 2000; Vogelstein and Kinzler, 1992). This process is another relevant mechanism responsible for its activity. The molecular mechanisms responsible for transcriptional repression have been less well elucidated. The ability of p53 to interfere with transcription factor Sp1 activity and binding to DNA has been discussed (Bargonetti and Manfredi, 2002).

1.3.2 THE p53 HOMOLOG p73

p53 was thought for a long time to be the only member of the p53 protein family. Recently, the identification of two p53-related proteins, termed p73 and p63 revealed that p53 belongs to a small family of sequence specific nuclear transcription factors (Kaghad et al., 1997).

p53 family members share the three major functional domains: the N-terminal transactivation domain, the central core sequence specific DNA binding domain highly conserved across the family, and the C-terminal oligomerization domain. The genomic organization of p73 is quite similar to that of p53. The p53 gene is 20 kb in length and contains 11 exons whereas the p73 gene is larger than 60 kb in length and contains 14 exons (Irwin and Kaelin, 2001). Unlike p53, though, p73 is expressed as at least 6 variants (p73 $\alpha, \beta, \gamma, \delta, \epsilon, \zeta$) with different C-terminal ends, arising from alternative splicing (Kaghad et al., 1997; Ueda et al., 1999). Among them p73 α is the longest form containing a sterile α motif domain (SAM domain) and an extreme C-terminal region while the p73 β lacks most of these two regions. Each of these splicing variants contains an intact N-terminal transactivation domain and exerts its transcriptional activity to various degrees, and the C-terminal differences among the variants attribute different transcriptional and biological properties. The C-terminal region of p73 might indeed possess a regulatory role which modulates its transactivation and pro-apoptotic activity (Ueda et al., 1999; Ozaki et al., 1999; Lee and La Thangue, 1999). These splicing variants are differentially expressed among normal tissues and cell lines, suggesting that they might have distinct physiological functions (Ueda et al., 1999). In addition to the differential splicing variants, p73

contains a second transcriptional starting site within intron 3, giving rise to the N-terminal truncated forms of p73 (Δ Np73 α and Δ Np73 β) with little transcriptional activity (figure 1.6) (Yang et al., 2000).

Δ Np73 displays a dominant negative behaviour toward p73 as well as wild type p53 and has oncogenic potential (Pozniak et al., 2000; Stiewe et al., 2002b). This inhibition occurs at the oligomerization level or by the competition for the binding to the same p53/p73 responsive elements as Δ Np73 would displace Tap73 and p53 from the DNA binding site (Stiewe et al., 2002a; Melino et al., 2002). A harmonic balance between the intracellular expression levels of pro-apoptotic Tap73 or p53 and anti apoptotic Δ Np73 might contribute to regulate cell fate determination.

It has been recently shown the existence of a functional p53/p73 responsive element within the Δ Np73 promoter region. The expression of Δ Np73, in fact, is directly transactivated by Tap73 and/or wt p53, creating a dominant negative feedback loop which regulates the activities of both Tap73 and wt p53 (Grob et al., 2001; Zaika et al., 2002). Recent finding attributed to Δ Np73 β but not to Δ Np73 α a weak but distinct transactivation activity, inducing cell cycle arrest and/or apoptosis (Liu et al., 2004). The expression levels of endogenous p73 are kept low under physiological conditions and, similar to p53, p73 is stabilized by post translational modifications at the protein levels in response to many DNA damaging agents resulting in either G1/S cell cycle arrest or cell death through apoptosis (Irwin et al., 2003). p73 stability is directly linked with its activity. Chemical modifications of p73 such as phosphorylation or acetylation would prolong its half life with enhancement of its

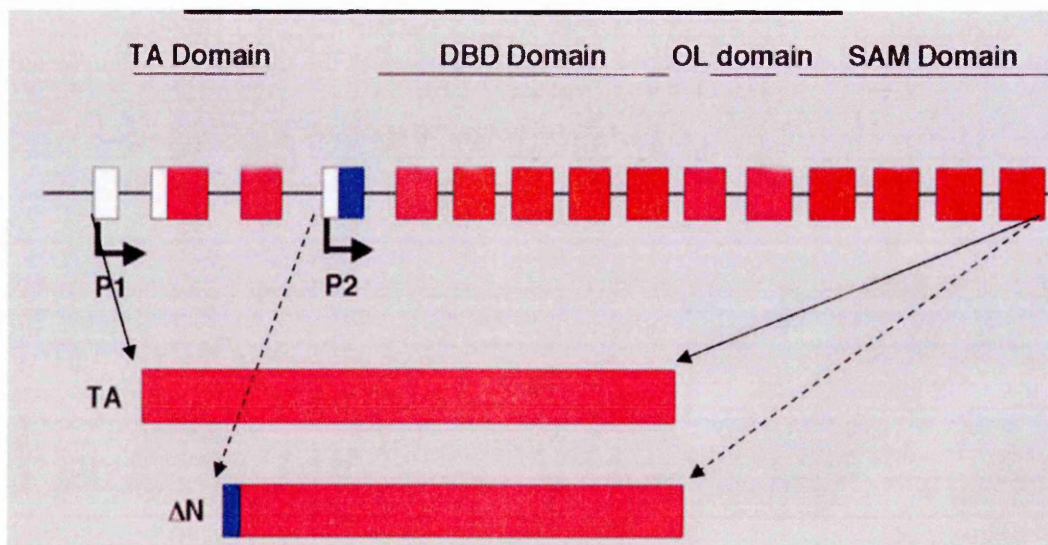


Figure 1.6 Structure of TA p73 and of Δ N p73

Schematic representation of the structure of TA and of Δ N isoforms of p73.

both transcriptional and pro- apoptotic activity. Experimental evidences suggest that p73 turnover is regulated through ubiquitination dependent and ubiquitination independent degradation pathway (Ozaki and Nakagawara, 2005). It has been shown that mdm2 interacts directly with the N-terminal transactivation domain of p73 promoting the p73 mediated transactivation activity and apoptosis without provoking the ubiquitination of p73 (Zeng et al., 1999). Thus, p73 stability seems to be regulated through a pathway distinct from that of p53, as also the evidence that the additional newly identified p53 induced E3 ubiquitin protein ligase pirh2 had a negligible effect on p73 (Leng et al., 2003; Wu et al., 2004). It has been recently shown that the HECT-type E3 ubiquitin protein ligase Itch, interacts with p73 through the WW protein-protein interaction domain of Itch and the p73 region with the PY motif and has the ability to ubiquitinate and degrade p73. The levels of this protein strongly decrease after DNA damage induced by different chemotherapeutic agents thereby increasing the stability and activity of p73. The same type of interaction was seen between p73 and a novel E3 ubiquitin protein ligase NEDL2. The NEDL2 mediated ubiquitination of p73 increased the stability of p73 and enhanced the p73 dependent transcriptional activity, thus indicating that a non proteolytic regulatory role of ubiquitination may exist (Rossi et al., 2005; Miyazaki et al., 2003).

Some studies demonstrated that the N-terminal truncated form of p73 (Δ Np73) is much more stable than Tap73, suggesting that p73 mediated transcriptional activation is required for the rapid turnover of p73 and that, like p53, one or more transcriptional targets of p73 might promote its proteolytic degradation (Wu et al.,

2004). Alternatively it was also reported that p73 may be degraded by ubiquitin independent manner through the binding to cyclin G, one of the direct transcriptional targets of p53 and p73, although the mechanism is yet to be defined (Ohtsuka et al., 2003).

1.3.2.1 P73 FUNCTION

As p73 has a p53 like property and is mapped in a chromosome region frequently lost or mutated in a wide variety of human tumors, it was thought that p73 could act as a tumor suppressor gene. Unlike p53 which is mutated in a high percentage of tumors, it was however found that p73 is infrequently mutated (p73 mutations detected in fewer than 0.5% of human cancers) (Kaghad et al., 1997; Ikawa et al., 1999). p73 deficient mice exhibit severe developmental defects but they do not develop spontaneous tumors, suggesting that p73 does not link directly to tumor suppression but may participate to the regulation of normal development *in vivo*, especially neural development and apoptosis (Yang et al., 2000). Some data support the evidence that in some way, p73 has an ability to enhance the activity of wild type p53. It has been shown recently that p53 dependent apoptosis requires the contribution of another p53 family member. P73 would cooperate with p53 to promote apoptotic cell death further emphasizing the functional importance of p73 in the regulation of the DNA damage induced apoptotic response. Some evidences strongly suggest that the pro-apoptotic activity of p73 would also be regulated through a pathway distinct from that one used for p53. This is further confirmed by

the evidence that p73 has the ability to promote apoptotic cell death in p53 deficient cells (Flores et al., 2002).

1.4 S-PHASE CHECKPOINT

DNA damage registered during S phase can interfere with the functioning DNA replication and may lead to genomic instability. For this reason cells developed the ability to detect the damage during S phase causing transient reversible inhibition of firing replicons that have not yet been replicated (Larner et al., 1997). Unlike the G1 or the G2-M checkpoint the S phase response to DNA damage is short and lacks the sustained maintenance phase of the cell cycle arrest; in fact cells that experience genotoxic stress during DNA replication only delay their progression through S phase in a transient manner and if damage is not repaired during this delay, once exiting S phase they arrest later during the G2 (Abraham, 2001; Bartek and Lukas, 2001; Shiloh, 2003). The main function of the S phase checkpoint is to maintain fork integrity, thus cell viability results as an indirect effect of prevention of DNA replication fork catastrophe (Tercero et al., 2003). Many different proteins participate to the S phase checkpoint activation. First of all it is possible to distinguish among replication independent and replication dependent S phase checkpoint (figure 1.7). The replication dependent intra S phase checkpoint, also commonly called replication checkpoint, is initiated when the progression of replication forks becomes stalled in response to stresses such as depletion of dNTP pools, chemical inhibition of DNA polymerases or as a consequence of collision of replication forks with damaged DNA or aberrant DNA structures. The key

components of this checkpoint include RPA, the ATR-ATRIP complex, the mediator claspin, Rad 17 and the 9-1-1 complex. This checkpoint acts to inhibit the initiation of DNA replication from unfired origins by targeting the cyclin-CDK and Cdc7-DBF4 kinases that regulate the assembly and firing of replication origins, and protect the integrity of the replication forks allowing the recovery of cell cycle progression after DNA repair or restoration of the dNTP pool (Nyberg et al., 2002; Lopes et al., 2001). Compounds such as hydroxyurea (HU) and aphidicolin (APH) trigger the replication checkpoint. HU is a ribonucleotide reductase inhibitor that leads to depletion of the small pool of cellular deoxyribonucleotide triphosphates while APH directly inhibits the activity of polymerase α . Treatment with these inhibitors leads to the accumulation of stalled replication forks and the activated S checkpoint acts to protect the replication fork from collapsing while DNA synthesis is stalled (Lopes et al., 2001). The replication independent intra S phase checkpoint does not require active replication forks for its initiation being activated by genotoxic agents such as IR or radiomimetic drugs that can cause DSB in the genome. Defects in the intra S phase checkpoint response to IR result in the incapability of cells to reduce the rate of DNA replication when irradiated, phenomenon known as radioresistant DNA synthesis (RDS) (Painter and Young, 1980; Merrick et al., 2004). Both ATM and ATR are required to prevent RDS thus activating the intra S phase checkpoint. ATM has a major role to sense DSB, as I already mentioned, (Shiloh, 2001) and a complex of three proteins, Mre11, Rad50 and Nbs1 termed the MRN complex, contributes to the recruitment of active ATM to sites of DSBs (van den et al., 2003; Lisby et al., 2004).

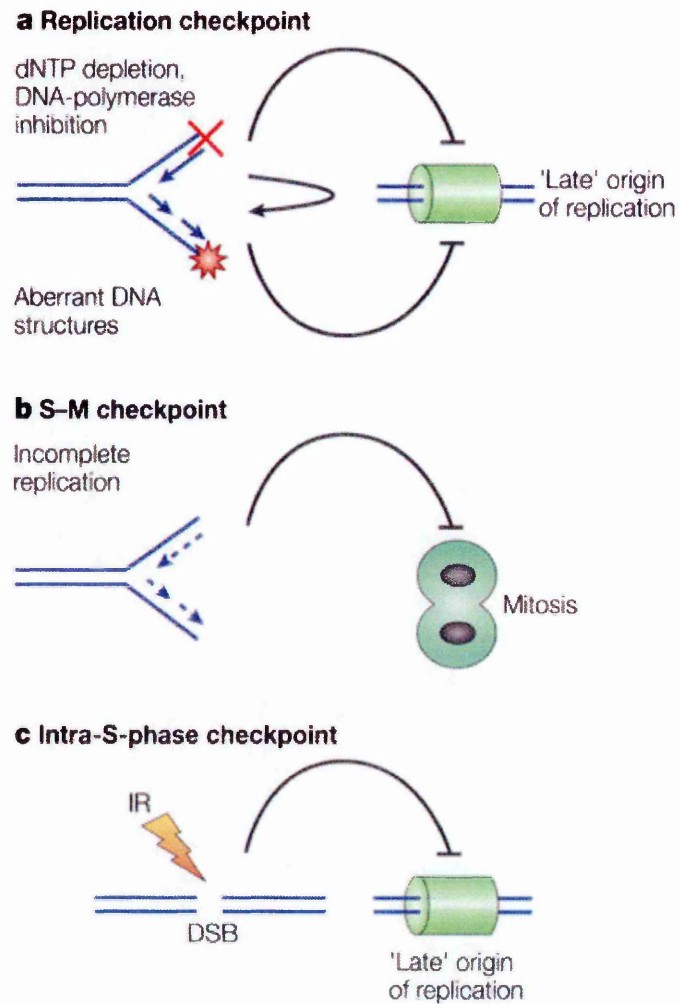


Figure 1.7 Activation of the S-phase checkpoint

Schematic representation of the distinct S phase checkpoint possibly activated by replicative stress (a), defects during DNA replication (b) and genotoxic agents (c).

(Modified by Bartek J. et al., 2004)

Mre11 presents an exonuclease activity while Rad50 and Nbs1 stimulate Mre11 enzymatic activity. Nbs1 also has a BRCA-1 C terminal domain (BRCT) responsible for protein-protein interaction. Once ATM is activated, it is present on the DSB sites, Chk2 gets activated diffusing rapidly throughout the nucleus (Lukas et al., 2003). Downregulation of both the ATM target effector kinase Chk2 and MRN are necessary for a complete recovery of radioresistant DNA synthesis thus suggesting that two parallel pathways (ATM-Chk2 and ATM-MRN) cooperate during the intra S phase checkpoint (Falck et al., 2002). Although the initial response to DSB is strictly ATM dependent, the ssDNA resulting from the processing of DSB promotes a slower activation of ATR which cooperates with ATM in the maintenance of the intra S checkpoint and in the inhibition of late origin firing (Shechter et al., 2004; Merrick et al., 2004). The ssDNA gets coated with replication protein A (RPA) which works as a template for the specific recruitment of the ATR interacting protein ATRIP-ATR heterodimer (Zou and Elledge, 2003).

As I previously mentioned both ATM and ATR exert their function through the cooperation and interaction of a class of checkpoint regulators called mediators. The proper timing and velocity of ATM controlled response, relies on the functional interplay of at least three mediators: MDC1, 53BP1 and BRCA1. It has been shown that BRCA1 can be phosphorylated by ATM or ATR. Furthermore ATR not only phosphorylates but also colocalizes with BRCA1 in nuclear foci during stalled replication (Gatei et al., 2000; Tibbetts et al., 2000). Nonetheless BRCA1 facilitates the ability of ATM or ATR to phosphorylate some downstream targets such as Chk2, p53 and Nbs1 and is also required for the activation of the effector kinase Chk1

(Foray et al., 2003; Yarden et al., 2002). Mediator of DNA damage checkpoint (MDC1) is another BRCT containing protein with a central role in the S phase checkpoint. It binds to pH2AX and is required for the recruitment of Nbs1 to broken DNA (D'Amours and Jackson, 2002; Lukas et al., 2004a; Petrini and Stracker, 2003). MDC1 also promotes the assembly of 53BP1 and BRCA1 into foci and facilitates the phosphorylation of SMC1 by ATR (Stewart et al., 2003; Yazdi et al., 2002). SMC1 is a component of the cohesion complex that is required for sister chromatid cohesion during S phase. It is predominantly phosphorylated by ATM in an Nbs1 and BRCA1 dependent manner (Yazdi et al., 2002; Kim et al., 2002). The 53BP1 (p53 binding protein 1) is important for the intra S phase checkpoint as it regulates the phosphorylation of Chk2, BRCA1 and SMC1 following IR (Wang et al., 2002; DiTullio, Jr. et al., 2002). In addition to these three proteins, signaling through the ATR controlled checkpoint involves claspin, another checkpoint mediator that specifically interacts with DNA structures generated by active replication forks (Lee et al., 2003; Kumagai and Dunphy, 2000). In mammals Claspin phosphorylation in response to DNA damage and replication stress results in recruitment and phosphorylation of BRCA1 and subsequent activation of Chk1 (Lin et al., 2004). The ATM/ATR downstream target kinases Chk1 and Chk2 once activated lead to the Cdc25A degradation resulting in the inhibition of the cyclinA/E-Cdk2 activity thus provoking the block of the loading of Cdc45 onto the chromatin and preventing subsequently the initiation of new origin firing (Bartek and Lukas, 2003; Bartek et al., 2004). To understand the regulation of the turnover of the Cdc25A into the intra S phase checkpoint it is necessary to underline that even during unperturbed S phase

Cdc25A is a relatively unstable protein with a half life of 20-30 minutes (Sorensen et al., 2003; Mailand et al., 2000; Falck et al., 2001; Molinari et al., 2000; Mailand et al., 2002). The basal turnover of Cdc25A requires ATR, claspin and especially Chk1 which through the phosphorylation of several serine residues in the regulatory domain of Cdc25A regulates the physiological levels of Cdc25A in unperturbed S phase preventing the accumulation of this powerful replication promoting activity (Sorensen et al., 2003; Zhao et al., 2002; Sorensen et al., 2004). After DNA damage, the proteolysis is accelerated by the additional phosphorylation of ser123 of Cdc25A by Chk2 which would amplify the physiological Chk1 dependent mechanisms (Bartek and Lukas, 2003; Sorensen et al., 2003).

The S-M checkpoint represents another additional type of S phase checkpoint. It ensures that cells do not try to divide before the completion of a faithful DNA duplication; its failure results in catastrophic mitosis of cells that have incompletely replicated their DNA. This checkpoint depends on defects occurring during DNA replication and the cyclinB1-Cdk1 complex is the key target of this mechanism (figure 1.7) (Bartek et al., 2004; Brown, 2003).

1.5 G2 CHECKPOINT

To ensure accurate segregation of chromosomes cells must prevent entry into mitosis in the presence of DNA damage. In fact, attempts to segregate either broken or unrepaired chromosomes, would lead to cell death or to a level of genomic instability with subsequent cell death or tumorigenesis. Cells have therefore evolved a G2 DNA damage checkpoint control that prevents cells from initiating mitosis when they

experience DNA damage during G2 or when they progress into G2 with some unrepaired damage inflicted during previous S or G2 phases (Nyberg et al., 2002; Xu et al., 2002). In normal conditions, the mitosis, the most spectacular phase of the cell cycle, is controlled by the cyclin B1-Cdk1 kinase activity (Nurse, 1990). Cdk1 activity, necessary to the G2-M transition, depends on the dephosphorylation of Thr14 and Tyr15 through the activity of the dual specificity phosphatase Cdc25C which neutralizes the effects of the opposite kinases Wee1 and Myt1 that keep Cdk1 in a phosphorylated form during interphase (Nigg, 2001). Cdc25C is normally localized in the cytoplasm and translocates to the nucleus just before mitosis (Russell, 1998; Raleigh and O'Connell, 2000). The mechanism responsible for translocation of Cdc25C between cytoplasm and nucleus implicates the association with 14-3-3 proteins (not the sigma isoform) (Dalal et al., 1999; Lopez-Girona et al., 1999). When the phosphatase is bound to 14-3-3 proteins, it remains cytoplasmic and the dissociation is necessary for nuclear import. The region of Cdc25C interacting with 14-3-3 proteins contains a phosphorylation site, serine 216, which has been shown to be indeed phosphorylated throughout interphase but not during mitosis (Ogg et al., 1994). The phosphorylation of serine 216 of Cdc25 is necessary for the association of this phosphatase with the 14-3-3 protein. The kinase responsible for the phosphorylation of Cdc25C on serine 216 during interphase has been cloned and named C-TAK1 (for Cdc Twentyfive C Associated protein Kinase) (Peng et al., 1998). C-TAK1 is ubiquitously expressed in the cytoplasm of human cells, where it would facilitate the phosphorylation of Cdc25C while it is being synthesised in the cytoplasm. Following stress induction, increased phosphorylation of Cdc25C is observed, which prevents activation of

Cdk1/cyclin B complex and arrests cells in G2 (Dalal et al., 1999; Smits and Medema, 2001). Depending on the kind of damage inflicted to the cell, Cdc25C gets phosphorylated on ser216 either by the nuclear checkpoint kinase Chk1 or Chk2 both previously phosphorylated and then activated by the upstream kinases ATM and ATR. This phosphorylation creates a binding site for 14-3-3 proteins (as I mentioned already) the association of which inhibits Cdc25C by causing its sequestration in the cytoplasm and/or masking some residues required for the interaction with cyclinB1-Cdk1 leading to the G2 block (Nyberg et al., 2002; Dalal et al., 1999; Kumagai and Dunphy, 1999; Morris et al., 2000). At first Cdc25C was thought to play the main role in regulating the G2-M checkpoint then subsequently it was found that Cdc25 Δ mice have a normal G2-M checkpoint (Peng et al., 1997; Chen et al., 2001). Recently it has been shown that the 2 other Cdc25 family members, Cdc25A and B are both implicated in the G2 DNA damage pathways in unperturbed cell cycle progression (Mailand et al., 2002; Donzelli and Draetta, 2003). Cdc25A gets stabilized in mitosis through phosphorylations on ser17 and ser115 by CyclinB1-Cdk1, thus uncoupling it from its ubiquitin proteasome mediated turnover (Mailand et al., 2002). Following DNA damage, Cdc25A gets degraded through a ubiquitin proteasome dependent pathway and its phosphorylation on residue of Thr507 by Chk1 provokes its cytoplasmic exclusion *via* 14-3-3 binding that in turn prevents its association with Cdk1 (Chen et al., 2003). This is basically what occurs also in the G1 and intra S phase checkpoint, making the Cdc25A pathway the most conserved mechanism shared by the three major checkpoints operating at G1-S, S, and G2-M (Bartek and Lukas, 2003; Donzelli and Draetta, 2003; Mailand et al., 2002; Xiao et

al., 2003). Experimental data evidenced that after UV, the mitogen activated kinase p38 gets activated and contributes to the initiation of the G2 checkpoint by phosphorylating Cdc25B at ser309 and 361, thus inducing the binding of the 14-3-3 protein which prevents the access of substrates to Cdc25B (Bulavin et al., 2001; Forrest and Gabrielli, 2001). Studies with specific inhibitors of p38 showed a decrease in initiation of G2/M checkpoint following UV radiation (Bulavin et al., 2001). This mechanism has been clearly observed following UV radiation, but not with other types of damage suggesting that it could represent an example of “damage specific” checkpoint activation. It has been shown that the protein kinase Wee1 also undergoes Chk1 dependent phosphorylation on residue ser549. This phosphorylation promotes the binding of the 14-3-3 protein that enhances the inhibitory activity of the kinase towards Cdk1 consolidating the G2 arrest upon damage (Lee et al., 2001; Rothblum-Oviatt et al., 2001).

The regulation of the cyclin B1 localization could also contribute to the G2-M checkpoint control. Cyclin B1 is initially localized in the cytoplasm during S and G2 phases and translocates to the nucleus at the beginning of mitosis (Pines and Hunter, 1991). The nuclear exclusion during S and G2 is due to the cytoplasmic retention signal at the N terminal part of the protein containing a hydrophobic nuclear export signal that binds the nuclear export factor CRM1 (Pines and Hunter, 1994; Li et al., 1995; Hagting et al., 1998). DNA damage leads to the retention of cyclin B1 in the cytoplasm and destruction of its nuclear exporting signal would compromise the DNA damage induced G2 arrest (Jin et al., 1998). Some studies suggest an additional regulation of the G2 block by the two members of the polo like kinases family

(PLK), Plk1 and Plk3 that play a role in several mitotic events like initiation and exit from mitosis and centrosomes function as well (Glover et al., 1998; Nigg, 1998). Interestingly the polo like kinase 1 seems to phosphorylate in ser147 cyclinB1 thus ensuring its nuclear translocation (Jackman et al., 2003; Toyoshima-Morimoto et al., 2001). Recent experimental evidences attribute to BRCA1 a role in the G2 checkpoint either through ATM/ATR or directly by activating Chk1. The relevance of this protein in this checkpoint is still under further investigation and clarifications (Tibbetts et al., 2000; Yarden et al., 2002; Larson et al., 1997; Somasundaram et al., 1997).

1.5.1 ROLE OF P53 IN THE G2 CHECKPOINT

Although p53 has been mainly involved in regulating the G1 checkpoint, it has also an important role in controlling the G2/M transition (Taylor and Stark, 2001). p53 is able to activate the transcription of three distinct genes interfering with the activity of Cdk1. It strongly activates the cdk inhibitor p21 which inhibits the Cdk1 kinase activity, although p21, as already discussed much less inhibit Cdk1 activity compared to other cdks (El Deiry et al., 1993). It has been described that p21 interacts with PCNA-Cdk1-cyclinB1, thus excluding Cdc25C from interacting with Cdk1 to dephosphorylate it for mitotic progression (Ando et al., 2001). p21 may also act by blocking CAK which activates Cdk1 through phosphorylation on thr161 (Pines, 1995b; Smits et al., 2000). p53 transcriptionally activates the GADD45a gene (growth arrest and DNA damage inducible 45 alpha), which is able to dissociate the complex between Cdk1 and cyclin B (Wang et al., 1999; Zhan et al., 1999). GADD45, which has specific activity against the

Cdk1/cyclin B complex, does not efficiently inhibit the activity of the CDK2/cyclin E complex. This fact is likely to be the reason why its role in G1 arrest is negligible (Zhan et al., 1999). Immunoprecipitation studies showed that GADD45 associates with Cdk1 and not with cyclin B, again indicating that it inhibits Cdk1 activity by preventing its binding to cyclin B (Zhan et al., 1999).

The third p53 downstream gene inhibiting Cdk1 activity is 14-3-3 sigma. This gene is particularly responsive to p53 *in vitro* (Hermeking et al., 1997). Its product, the protein 14-3-3 sigma, inhibits Cdk1 activity with a mechanism distinct from those activated by p21 or GADD45. The Cdk1/cyclin B complex needs to be present in the nucleus to exert its activity and to activate mitosis, through a mechanism involving the binding of importin alpha and beta. The 14-3-3 σ protein is able to bind Cdk1 and to anchor the complex Cdk1/cyclin B in the cytoplasm (Taylor and Stark, 2001; Chan et al., 1999). Prevention of nuclear translocation of Cdk1/cyclin B is sufficient to halt the cells in G2. An additional p53 dependent mechanism of G2 arrest is the direct repression of cyclin B and Cdk1 gene transcription. As already discussed (see chapter 1.3.1.2) not only p53 activates the transcription of different genes through direct binding to a recognition DNA sequence, but it can also repress the transcription of different genes through several distinct mechanisms, including squelching of transcription factors, binding to proteins, such as the histone acetyl transferase p300 necessary for transcriptional activation of certain genes interfering with sequence specific transcription factors (Levine, 1997). Among the genes repressed by p53 is cyclin B1 (Innocente et al., 1999; Krause et al., 2000). The cyclin B1 promoter region responsible for these effects of p53 has been mapped. Even for Cdk1 there are data suggesting a direct transcriptional

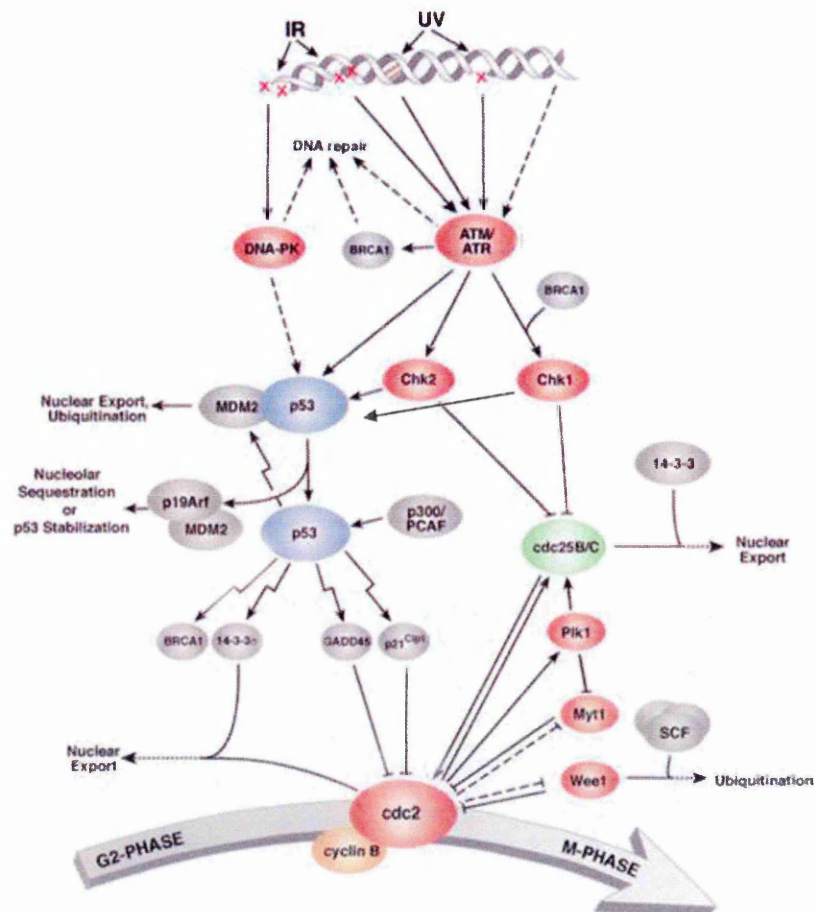


Figure 1.8 G/M DNA damage checkpoint: molecular players

Schematic representation of the principal molecular mechanisms involved in the regulation of the G2-M transition following DNA damage.

repression by p53, and a promoter region of the Cdk1 gene has been mapped and shown to be susceptible to p53 (Yun et al., 1999; Taylor et al., 2001).

It has been observed that many cell types that lack p53 still have the ability to accumulate in G2 after DNA damage thus indicating that redundant p53 independent mechanisms such as the BRCA1 dependent expression of p21 and GADD 45 may cooperate with the p53 pathway to sustain the G2 arrest (Zhan, 2005). This phenomenon has indeed inspired efforts to interfere with the G2 checkpoint as a potential strategy to sensitize cancer cells deficient in their G1-S checkpoints pathways to radiation or drug induced DNA damage, as I will further discuss in the chapter 1.9.

In figure 1.8 the principal components involved in the G2 checkpoint activation and just described are summarized.

1.6 MITOTIC SPINDLE CHECKPOINT

During mitosis, the two daughter cells have to receive just one copy of each chromosome. In this phase microtubules organize into a bipolar spindle which segregates the duplicated chromosomes (Meier and Ahmed, 2001). A specialized DNA-protein complex, known as the kinetochore, allows the attachment of sister chromatids to microtubules emanating from opposite poles of the mitotic spindle. When all kinetochores have bound microtubules, the mitotic spindle can proceed to pull the sister chromatids apart. This process must be tightly controlled because mis-segregation of chromatids will lead to aneuploidy (Jallepalli and Lengauer, 2001).

A surveillance mechanism known as spindle checkpoint is essential to ensure fidelity on chromosome transmission. This checkpoint monitors the correct attachment of kinethocores to microtubules and inhibits sister chromatids separation, thus onset of anaphase, when a defect is detected (Straight, 1997; Allshire, 1997).

The crucial steps in the progression through mitosis are controlled by the disruption of the mitotic inhibitory proteins, a phenomenon occurring when these proteins are ubiquitinated by APC/C (anaphase promoting complex/cyclosome) and targeted to the proteosome for degradation (Morgan, 1999; Harper et al., 2002). Basically, just before the end of mitosis the APC ubiquitin ligase coupled to the Cdc20 adaptor protein, becomes phosphorylated and thereby activated, by cyclin CDK enzymes. Together with ubiquitin carrier proteins (Ubc) the activated APC transfer Ub to several substrates including cyclinA and B whose destruction is necessary to complete DNA segregation. The two cyclins thus trigger to a chain of events that lead to their own destruction which is necessary to initiate the new cell cycle. At the beginning of the following G1 phase, the APC swaps its substrate binding adaptor from Cdc20 to Cdh1 maintaining APC activated to allow complete destruction of mitotic regulators of the previous cycle and avoiding their premature accumulation. Before S phase begins, Cdh1 is phosphorylated and dissociates from APC which gets inactivated re allowing accumulation of APC substrates required for DNA synthesis and next mitotic entry thus closing the cycle (Lukas and Bartek, 2004). The mitotic checkpoint directly inhibits the APC/C function (Wassmann and Benezra, 2001). Genes able to signal and activate this checkpoint have been discovered and include the Mad and Bub genes which have been well characterized in *Saccharomyces. cerevisiae*.

Mammalian homologs have been recently discovered and also found to play a similar role in the mitotic checkpoint (Li and Benezra, 1996; Taylor et al., 1998).

1.7 CHK1 AND CHK2: TWO KEY CHECKPOINT REGULATORS

The mammalian kinases Chk1 and Chk2 are two critical messenger of the genome integrity checkpoints well conserved throughout the eukaryotic evolution. They are structurally unrelated serine/threonine kinases activated in response to diverse genotoxic insults, playing some redundant role by sharing a wide spectra of known substrates involved in the activation of the DNA damage checkpoint response (Bartek and Lukas, 2003).

1.7.1 CHK1

The human checkpoint kinase 1 (Chk1) gene was identified in 1997 by Sanchez et al. by a degenerated PCR strategy, basing on the already identified gene encoding Chk1 in *Saccharomyces pombe*, finding out a very high similarity of this checkpoint gene among the eukaryotic kingdom (Sanchez et al., 1997). The function of Chk1 has been studied both in *S. pombe* and mammalian cells and found to be similar. In *S. pombe*, Chk1 is essential for cell cycle arrest following DNA damage (Lindsay et al., 1998) by activating an analogous checkpoint pathway involving the homologs of the mammalian ATM/ATR, and Cdc25C as I will describe in the next paragraph.

The chromosomal location of Chk1 was mapped to 11q24 by fluorescence in situ hybridization. That is a region adjacent to the one containing the gene encoding

ATM at 11q23 presenting frequent deletions and LOH (loss of heterozygosity) in a numbers of cancers including breast, ovary and lung. The human protein encoded by a cDNA of 1,891 bp, is a nuclear protein of 476 amino acids with a molecular size of 54 KDa. The predicted human Chk1 protein is 29% identical and 44% similar to *S. pombe* Chk1, 40% identical and 56% similar to *Caenorhabditis elegans* Chk1, and 44% identical and 56% similar to *Drosophila* Chk1 (Sanchez et al., 1997). Northern blot analysis revealed ubiquitous expression of human Chk1, with large amounts in human thymus, testis, small intestine and colon (Sanchez et al., 1997). Chk1 protein is expressed specifically at the S to M phase of the cell cycle (Kaneko et al., 1999) and is absent or expressed at very low levels in quiescent and differentiated cells (Kaneko et al., 1999; Lukas et al., 2001). It is activated even in unperturbed cell cycles although it is further activated after DNA damage or stalled replication (Sorensen et al., 2004; Zhao et al., 2002; Kaneko et al., 1999) and its activation does not require Chk1 dimerization and autophosphorylation.

Mutations in the Chk1 gene are rare in human tumors (than those found with respect to other checkpoint genes such as Chk2). The presence of a nucleotide stretch of nine consecutive adenines in the coding region of Chk1 led to the hypothesis that tumors with defects in mismatch repair and hence with microsatellite instability could accumulate mutations around this region. This hypothesis was indeed found to be correct. In tumors of the colon and endometrium, in which microsatellite instability occurs frequently, insertion/deletion of one nucleotide in the coding region of Chk1 could be found (Bertoni et al., 1999; Vassileva et al., 2002; Furlan et al., 2002). The insertion/deletion of one nucleotide leads to a frameshift mutation that in the case of CHK1 results in a

protein inactivated and truncated of 243 aa (polyA10) or 238 aa (polyA8) in comparison with the normal length of 476 aa (Bertoni et al., 1999). Those truncated forms are predicted to be defective due to the lack of the C-terminal end of the catalytic domain and the complete loss of the SQ-rich regulatory domain. The mutations found were always heterozygous and one wt allele was always present. A shorter isoform of Chk1 mRNA, predicted to encode a protein which lacks a conserved subdomain in the catalytic domain of Chk1 which is involved in substrate specificity, has been also detected in a subset of small cell lung carcinomas (Haruki et al., 2000). Sequence analysis revealed a highly conserved N-terminal kinase domain (residues 1-265), a flexible linker region and a less conserved C-terminal region with undefined function (figure 1.9).

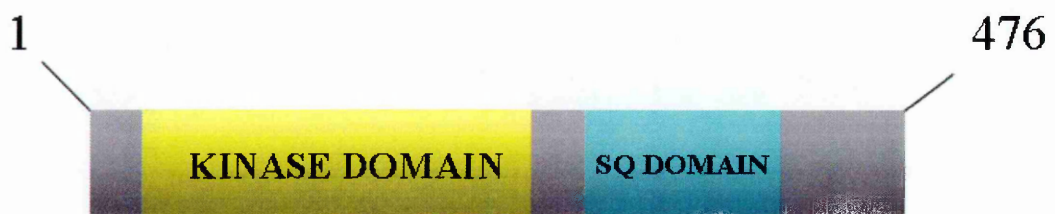


Figure 1.9 Structure of human Chk1 gene

The kinase domain and the serine/glutamine domains are indicated. N and C indicate the amino and carboxy terminal respectively.

The crystal structure of the human Chk1 kinase domain (residues 1-289) revealed structural features important for kinase activity and substrate selectivity, suggesting that Chk1 becomes fully active upon substrate binding, and that its activity is not regulated by phosphorylation within the kinase domain (Chen et al., 2000). The crystal analysis also suggested a potential regulatory role of the less conserved C terminal region of Chk1 which could control the access of substrates to the Chk1 kinase domain by interacting with the substrate binding site at the front of the kinase domain (Chen et al., 2000). An oriented peptide library approach could be utilized to define the consensus substrate motifs for Chk1 kinase protein, as well as for Chk2, as they share several substrates (O'Neill et al., 2002). Several independent peptide libraries each containing over 10^8 potential substrates targets were analyzed and gave the following consensus sequence motif preference: Φ -X- β -X-X-(S/T)- Φ . Φ in position -5 and +1 has to be a hydrophobic residue playing an important anchoring function to the kinases catalytic pocket according to the crystal Chk1 structure (Chen et al., 2000; O'Neill et al., 2002); X can be any aa residue and β at position -3 would be better be a basic aa especially either R or K (O'Neill et al., 2002; Hutchins et al., 2000). In terms of relative preference for Chk1 and Chk2 substrates, positions -3>-5>>+1.

1.7.1.1 CHK1 FUNCTION

Chk1 was originally identified in fission yeast (*S. pombe*) by genetic studies as a protein kinase required to delay entry of cells in mitosis after DNA damage (Walworth et al., 1993; al Khodairy et al., 1994; Walworth and Bernards, 1996).

DNA damage-induced mitotic delay was ascribed in fission yeast primarily by a inhibitory Tyr15 P of Cdk1 (O'Connell et al., 2000). In response to DNA damage Chk1 is activated in a Rad3-dependent manner (yeast homolog of mammalian ATM and ATR) (Lopez-Girona et al., 2001), preventing the activation of Cdk1 by inhibiting Cdc25 phosphatase, a Cdk1 activator (Rhind et al., 1997; Furnari et al., 1999), and stimulating the activity of the Mik tyrosine kinase, a Cdk1 inhibitor (Baber-Furnari et al., 2000; Christensen et al., 2000).

In mammalian cells, several experimental data have provided biochemical evidence that as in fission yeast Chk1 participates in the G2/M DNA damage checkpoint by phosphorylating and modulating the activity of the Cdk1 regulators Cdc25C phosphatase and Wee1 kinase, relatives of S pombe Cdc25 and Mik1 respectively (Rhind and Russell, 2000). Additional evidences for an *in vivo* role for Chk1 in the mammalian G2/M DNA damage checkpoint come from the observation that Chk1 deficient embryonic cells (ES) die of p53-independent apoptosis showing a defective G2/M checkpoint (Liu et al., 2000; Takai et al., 2000). Furthermore, analysis of Chk1 deficient mouse blastocysts revealed that Chk1 is indispensable in mammals for proper embryogenesis. Chk1 null mice, in fact, exhibit embryonic lethality (E6.5) due to a preimplantation defect, and this lethality was not rescued in a p53 null background (Liu et al., 2000; Takai et al., 2000).

In general, introducing Chk1 in the DNA damage transducing signal cascade, as I just described, it has to be underlined that it is phosphorylated on ser317 and ser345 by ATR after UV and replicative stress and these phosphorylation events are required to amplify the signal to downstream targets (Sorensen et al., 2003; Zhao et al., 2002).

ATR mediated phosphorylation of Chk1 requires the DNA binding protein claspin, which may be responsible of the recruitment of Chk1 to the DNA lesions where ATR resides (Kumagai and Dunphy, 2000). The main Chk1 target proteins are the Cdc25 phosphatases which regulate the cell cycle progression by activating the cyclin dependent kinases (CDKs) (Sanchez et al., 1997; Furnari et al., 1997). Chk1 mediated phosphorylation and inhibition of Cdc25 phosphatases (thereby cdk's) has been implicated in cell cycle checkpoint control of G1/S-S-G2/M phases as I mentioned in the previous sections (Sorensen et al., 2003; Zhao et al., 2002; Mailand et al., 2000; Mailand et al., 2002; Sanchez et al., 1997; Furnari et al., 1997). Recently it has been shown that the standard parallel subdivision of Chk1 activation by ATR after UV and replicative stress and by ATM after IR exposure is not valid anymore. In fact, in a recent paper (Jazayeri et al., 2006) it was shown that in response to IR, ATM and ATR function in the same pathway leading to Chk1 phosphorylation. ATM and its regulatory partner Nbs1 would promote DSB induced ATR dependent Chk1 phosphorylation by regulating the formation of RPA coated ssDNA that is required for ATR recruitment to sites of DNA damage. These events also require Mre11 nuclease activity implying another link between ATM and ATR and new important implications of the understanding of how checkpoint activation is coordinated with DSB repair. It is to mention that data also attested a connection between Chk1 and the homologous recombination repair protein rad51 (Sorensen et al., 2005).

Recent data showed that after Chk1 activation by agents that induce replicative stress, such as CPT, a time dependent down regulation of the activated protein subsequently occurred in both normal and transformed cell lines. Basically the ATM

dependent phosphorylation in ser345 would serve also as a target for subsequent Chk1 polyubiquitination and proteasomal degradation thus limiting the duration of Chk1 signaling during low intensity replication stress and preventing the active protein kinase from accumulating to levels that would be deleterious to normal S phase progression. The targeted destruction of activated Chk1 may have the meaning to limit the duration of ATR dependent S phase checkpoint during the normal cell cycle. The temporal delay between Chk1 activation and the onset of Chk1 ubiquitination is due to the time required for the mobilization of activated Chk1 from chromatin to the soluble nuclear compartment where Chk1 meets the Cull1/cul4a complex containing E3 ligases. The delayed degradation of Chk1 presumably allows sufficient time for the activated protein kinase to exert its protective effect at stalled forks prior to its modification by the E3 ligases (Zhang et al., 2005).

1.7.1.2 ROLE OF CHK1 IN CHECKPOINTS ACTIVATION AND CELL PHYSIOLOGY: NEW EXPERIMENTAL SYSTEMS LACKING CHK1

In these past few years a lot of research has been focused on the attempt to create experimental systems lacking the Chk1 function in order to better understand its role in the cell physiological context to provide a clearer insight in its involvement in the DNA damage checkpoints. RNAi mediated knockdown of Chk1 in human cells confirmed its requirement in the G2-M checkpoint in response to IR and some other DNA damaging agents (Gatei et al., 2003; Xiao et al., 2003) and revealed an essential role of this kinase in the control of Cdc25A protein turnover in both normal S phase and the intra S phase DNA damage checkpoint (Sorensen et al., 2003; Zhao

et al., 2002). A nice contribution to the understanding of the role of Chk1 in somatic vertebrate cells was supported by Zachos et al. with the construction by gene targeting of the avian B-lymphoma DT40 cell line Chk1 $-/-$ (Zachos et al., 2003). Chk1 is not essential for the viability of vertebrate somatic cells under normal culture conditions, similarly to what it was observed in yeast, although they grow slowly probably because of an increase incidence of apoptotic cell death (p53 independent as these cells do not have p53). This is in contrast to what have been observed in early development in both mouse and *Drosophila*, suggesting additional specific functions for Chk1 during embryonic development no longer required in somatic cells. The avian Chk1 $-/-$ cell line fails to arrest in G2 after exposure to IR, supporting the previous observations just described and confirming a crucial role for Chk1 in activating the G2/M checkpoint after DNA damage. These Chk1 deficient cells were more sensitive to DNA damage agents and DNA synthesis inhibition, showing that some Chk1 dependent processes promote cell survival after DNA structure or metabolism perturbations. In order to elucidate the role of Chk1 in the S-M checkpoint control in vertebrate cells, the effect of DNA synthesis inhibition on mitotic entry was investigated in the Chk1 $-/-$ DT40 cell line. It was observed that cells deficient in Chk1 enter mitosis with incompletely replicated DNA, when DNA synthesis is inhibited, but only after an initial delay. This mitotic entry is associated with an induction of Cdk1 catalytic activity which occurs in the absence of any loss of Cdk1 tyr15 phosphorylation. Mitotic entry from S phase would be due to the loss of the capacity to synthesize DNA even though DNA replication in these cells remains incomplete (Zachos et al., 2005) probably because of the degeneration of

viable replication structures which are normally stabilized by Chk1 during replication arrest (Zachos et al., 2003; Feijoo et al., 2001). Chk1 would so maintain the S-M checkpoint indirectly by stabilizing viable replication structures whose presence contributes to delay mitosis in vertebrate cells until DNA replication is complete.

Additional observations were conducted on DT40 Chk1 $-/-$ after the release from a shorter replication arrest, consisting of 8h exposure with aphidicolin instead of 12h. At first it was observed that the shorter replication block did not inhibit the S-M checkpoint in Chk1 $-/-$ cells that delay the mitosis as well as wt cells. Moreover after aphidicolin release the majority of Chk1 $-/-$ cells fails to enter mitosis, being unable to recover from replication arrest, which had caused DNA damage. It was also shown that after release, the replication forks which were active prior to aphidicolin treatment in Chk $-/-$ cells got progressively unable to resume replication and new sites of replication, futile origin firing, progressively accumulated. This was not observed in the wt cells providing a genetic proof that Chk1 is essential to maintain the viability of stalled replication and suppress origin firing when DNA synthesis is inhibited (Zachos et al., 2003). Taken together these evidences demonstrate that Chk1 is essential for the recovery from replication arrest and that in contrast to its secondary role in fission yeast (Sanchez et al., 1999), Chk1 dependent replication checkpoint functions are important determinants of vertebrate cell survival under conditions of DNA synthesis inhibition. These data further confirm observations regarding the putative role played by Chk1 on replication checkpoint, as for example the observation that *Xenopus* Chk1 is activated by replication arrest and is required

to prevent premature mitosis in egg extracts *in vitro* (Kumagai et al., 1998; Michael et al., 2000). Chk1 deficient mouse blastocysts were found to undergo premature mitosis when DNA synthesis was inhibited with aphidicolin suggesting a role of Chk1 in S-M checkpoint *in vivo* (Takai et al., 2000). Furthermore, a selective inhibitor of Chk1 (UCNO1) impaired the replication fork viability and disturbed the control of origin firing in mammalian cells when DNA synthesis was inhibited (Takai et al., 2000; Feijoo et al., 2001).

To investigate the role of Chk1 in adult tissue and its role in tumorigenesis, a mouse conditional KO for Chk1 in the mammary gland was generated (Lam et al., 2004). It was observed that Chk1 was essential for mammary epithelial cell growth; in addition the phenotype appeared strong also in the heterozygous background suggesting a possible role of Chk1 (-/+) as a haploinsufficient tumor suppressor. Briefly in the haploinsufficient tissues it was observed an inappropriate entry into S phase, an accumulation of DNA damage during replication and a failure to restrain mitotic entry in the presence of damaged S phase. The inappropriate entry in S phase could be explained by a possible role played by Chk1 in regulating the G1-S transition through its direct control of Cdc25A protein levels. With lower levels of Chk1 cells could inappropriately exit G1 because of the increase of Cdc25A levels which accelerate the G1-S transition through a premature activation of cyclin E and A dependent kinases. The accumulation of spontaneous DNA damage during S phase was underlined by the accumulation of a high number of foci containing MDC1, 53BP1 and pH2AX proteins in the Chk1+/- tissues. Physiological Chk1 protein levels could play a critical role in the regulation of DNA replication (Feijoo et al.,

2001; Lee et al., 2003) thus haploinsufficiency for this function would lead to inability to properly repair replication problems resulting in the accumulation of damage at replication structures, further corroborating the already discussed data on Chk1's critical role in DNA replicational stress (Zachos et al., 2003; Zachos et al., 2005; Feijoo et al., 2001). The increased levels of Cdc25A in haploinsufficient systems can override the restraint of mitotic entry during S phase, by activating Cdk1/CyclinB complexes during S phase, which causes the inhibition of PP1 (protein phosphatase 1) and the accumulation of Aurora B driving cells into mitosis (Goto et al., 2002; Sugiyama et al., 2002). These data would suggest for Chk1 a role as a haploinsufficient tumor suppressor and imply that inactivation of a single Chk1 allele in human tumors by mutation, chromosomal deletion, or epigenetic silencing may be a common source of genomic instability driving tumorigenesis.

To further corroborate these last observations, recently Bartek J. and his group showed in Chk1 inactivated human cells (by siRNA or inhibitors) how Chk1 plays an important role during normal S-phase progression by minimizing the occurrence of aberrant replication-associated events (Syljuasen et al., 2005). In response to Chk1 inhibition, Cdc25A is stabilized leading to an increase of CDKs activity related to increased initiation of DNA replication, measured by increased loading of Cdc45 protein to chromatin and an increased rate of DNA synthesis. This is accompanied by an increased binding of RPA to ssDNA with subsequent ATR activation and phosphorylation of its targets. During this process DNA is destabilized leading to formation of DNA strand breaks. An alternative possibility is that in response to Chk1 inhibition, replication forks may collapse due to a lack of Chk1 mediated

maintenance of stalled replication forks during normal S phase progression, thus resulting in the formation of ssDNA and DNA breaks and phosphorylation of ATR targets (Syljuasen et al., 2005). These data would suggest that these human cellular models behave differently from the highly genetically unstable chicken cells used in Zachos's experiments.

1.7.1.2.1 Chk1, centrosomes and mitotic entry control

It has been recently observed that the Chk1 protein localized not only in the nucleus but also in the centrosomes of interphase cells suggesting a new Chk1 involvement in the regulation of mitotic entry in unperturbed cell cycle progression (Kramer et al., 2004). According to these data, Chk1 dissociates from centrosomes at the onset of prophase concomitant with centrosomes separation. As Chk1 controls Cdk1 activity during normal unperturbed cell cycle progression (Hu et al., 2001) and the disappearance of Chk1 from centrosomes correlates with Cdk1 activation, it is likely that a centrosome associated pool of Chk1 works as a negative regulator of centrosomal Cdk1. It was previously observed that Cdk1-cyclinB1 accumulates at the centrosomes during interphase where initial activation of the complex occurs in late prophase (Bailly et al., 1989; Bailly et al., 1992; Jackman et al., 2003). Those are the initial activation of Cdk1 by dephosphorylation of tyr15 (De Souza et al., 2000) and of Cyclin B by phosphorylation of ser126 and ser133 (Jackman et al., 2003). Experimental evidences attributed to Cdc25B phosphatase the role of the initial activation of the mitotic complex Cdk1/CyclinB1 at the centrosomes during unperturbed cell cycle progression. In fact this phosphatase has been reported to

localize to the centrosomes during mitosis after being phosphorylated on ser353 by aurora A kinase (Dutertre et al., 2004). Basically the idea is that positive and negative pathways for the initiation of mitosis are integrated at the centrosomes with Cdc25B-CyclinB Cdk1 acting as common downstream targets and Aurora A and Chk1 upstream positive and negative regulators respectively. During interphase centrosomal Chk1 would keep the complex in an inactivated form by negatively regulating Cdc25B; at the onset of prophase, when Chk1 is detached from centrosomes, Cdc25B is phosphorylated and activated by the centrosomal Aurora A thus ensuring mitosis by its recruitment in the centrosomes and subsequent activation of the centrosomal Cdk1-cyclinB1 (Dutertre et al., 2004).

These evidences indicate that mammalian centrosomes might have an important role in regulating cell cycle progression from G2 phase into mitosis in addition to the already postulated functions in cytokinesis and G1/S transition.

1.7.1.2.2 Biology of centrosomes

The centrosome is a small non membranous organelle (1-2 μm in diameter) normally localized at the periphery of nucleus. Its primary function is to anchor microtubules, in fact it is often denoted as a major microtubule organizing center (MTOC). In animal cells the centrosome consists of a pair of centrioles and a number of different proteins surrounding the centriole pair called pericentriolar material (PCM) (Paintrand et al., 1992).

The centrioles differ from each other as one of those present a set of appendages at the distal ends (mother centriole) while the other one does not have appendages

(daughter centriole). The appendages appear to be essential for anchoring microtubules and the daughter centriole acquires them in late G2 phase of the cell cycle (Bornens, 2002).

During interphase centrosomes organize the cytoplasmic microtubule network which is involved in vesicle transport, proper distribution of small organelles, control of cellular shape and polarity.

During mitosis, centrosomes become the core structures of spindle poles and direct the formation of bipolar mitotic spindles which is an essential event for accurate chromosome segregation into daughter cells.

Since each daughter cell receives only one centrosome upon cytokinesis, the centrosome, like the DNA, must duplicate once prior to the next mitosis. DNA and centrosomes represent the only two organelles that undergo semiconservative duplication once every single cell cycle.

At this regard, animal cells are equipped with a mechanism that coordinates these two events, likely to ensure these two organelles to duplicate only once (Mazia, 1987).

Centrosome initiates duplication by physical separation of the paired centrioles at G1/S transition.

In the proximity of each pre existing centriole a procentriole progressively elongate during S and G2 and two centrosomes continue to mature by recruiting PCM. By late G2 two mature centrosomes are generated (figure 1.10).

The initiation of DNA and centrosomes duplication is coupled by the activation of CDK2/cyclinE (Lacey et al., 1999; Matsumoto et al., 1999). The presence of more

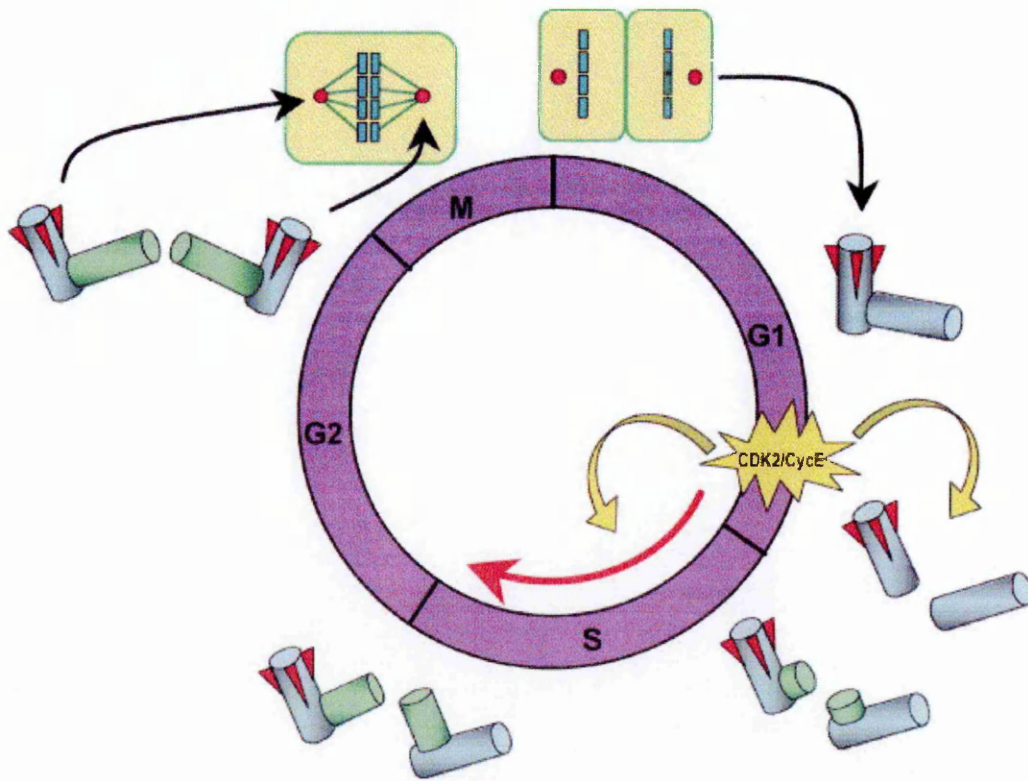


Figure 1.10 Centrosomes duplication

Schematic representation of the centrosomes duplication cycle of every single cell cycle. (Modified by Fukasawa, 2005)

than two centrosomes (centrosome amplification), severely disturbs mitotic progression and cytokinesis *via* formation of more than two spindle poles, resulting in an increased frequency of chromosome segregation errors (chromosome instability). Destabilization of chromosomes by centrosome amplification aids acquisition of further malignant phenotypes, thus promoting tumor progression (Fukasawa, 2005).

1.7.2 *CHK2*

Chk2 was at first identified in 1994 in budding yeast as a kinase involved in many checkpoint responses and called Rad53 (Allen et al., 1994). Homologs of Rad53 were subsequently found in the fission yeast and in higher eukaryotes (Murakami and Okayama, 1995; Brown et al., 1999). The overall structure of Chk2 proteins is similar in all eukaryotes with a degree of homology of the protein sequence across species that roughly reflects the evolutionary distance among the different organisms. The human protein encoded by the human cDNA of 1,731 bp, is a nuclear protein of 543 amino acids with a molecular size of 60 KDa. The Chk2 protein consists of three distinct functional domains (figure 1.11A). The N-terminal domain contains the SQ/TQ cluster domain (SCD, residues 19-69) with five SQ and two TQ motifs which satisfy the primary substrate motif for ataxia-telangiectasia mutated (ATM) kinase (Kim et al., 1999), especially the residues 67-70 STQE containing the key phosphorylatable site Thr68 (O'Neill et al., 2000). The central forkhead associated domain (FHA, residues 112-175), conserved from yeast to human, consists of 11 beta sheets forming a structural core and connecting loops conferring diversity in phosphopeptide recognition. It works

in *trans* to modulate protein-protein interactions but also in *cis* to affect other functional domains within the protein itself (Li et al., 2000). The C-terminal Ser/Thr kinase domain (residues 220-486) which shares homology to other Ser/Thr or Tyr kinases, related to the Ca²⁺/ calmodulin dependent protein kinase (CaMK) family. Differently to the expression of Chk1 restricted to S-G2-M phases, Chk2 remains stably expressed and can become activated upon DNA damage in all phases of the mammalian cell cycle as well as in non proliferating and terminally differentiated or quiescent cells. In humans Chk2 is homogeneously or heterogeneously nuclear expressed in all the tissues with the exception of neuronal cells with a predominant cytoplasmic localization (Lukas et al., 2001). Accumulating experimental evidences attributed to Chk2 a role of tumor suppressor. The first indication of a tumor suppression function for Chk2 came from the finding of rare germline mutation in the Chk2 gene in families with Li Fraumeni syndrome (LFS), a familial cancer predisposition syndrome usually associated with germline p53 mutations (Evans and Lozano, 1997). This Chk2 mutation confers increased breast cancer risk independent of mutation in the breast cancer susceptibility associated loci (BRCA) (Vahteristo et al., 2002; Meijers-Heijboer et al., 2003; Meijers-Heijboer et al., 2002). Several other missense mutations could be found in all three functional domains in a variety of tumor types (Sodha et al., 2002; Schutte et al., 2003) with different functional impact depending on the location of the mutation.

1.7.2.1 ACTIVATION AND FUNCTION OF CHK2

Chk2 gets largely activated following DNA damage induced by IR and different DNA damaging agents. ATM is mainly involved in its activation as multiple lines of

evidence suggested (Matsuoka et al., 1998; Brown et al., 1999; Chaturvedi et al., 1999). ATM directly phosphorylates Chk2 at thr68 *in vitro* and alanine substitution of this residues prevented activation of Chk2 after IR *in vivo* (Ahn et al., 2000; Melchionna et al., 2000). Chk2 may also be activated by ATR after high levels of damage, UV and HU (Matsuoka et al., 2000). The inactive Chk2 exists as a monomer and upon DNA damage it gets phosphorylated in thr68 and undergoes dimerization with a region spanning phosphorylated Chk2 at thr68 in one Chk2 molecule binding to the FHA domain of second molecule (figure 1.11B) (Ahn et al., 2002; Ahn and Prives, 2002). Dimerization is followed by multiple intermolecular phosphorylation events including thr383 and thr387 within the autoinhibitory loop resulting in kinase activation (Lee and Chung, 2001). The phosphorylation in thr68 appears to be necessary for the first dimerization and activation while its maintenance is not required for sustained kinase activity (Ahn and Prives, 2002), although it appears to be important for the association of other checkpoint proteins as for example MDC1.

As I just described for Chk1, an oriented peptide libraries analysis allowed to picking up the phosphorylatable consensus motif of Chk2 which is basically the same as the one described for Chk1. The main Chk2 substrates are the two members of the cell division cycle 25 dual specificity phosphatase family, Cdc25A and Cdc25C. Cdc25A is destabilized by Chk2 phosphorylation at ser123 (Falck et al., 2001; Sorensen et al., 2003) most likely by increasing susceptibility to the beta SCF^{trcp} complex which mediates Cdc25A turnover (Busino et al., 2003; Jin et al., 2003). Chk2 phosphorylation of ser216 in Cdc25C creates a binding site for 14-3-3 proteins resulting in persistent cytoplasmic Cdc25C localization thus preventing the G2-M

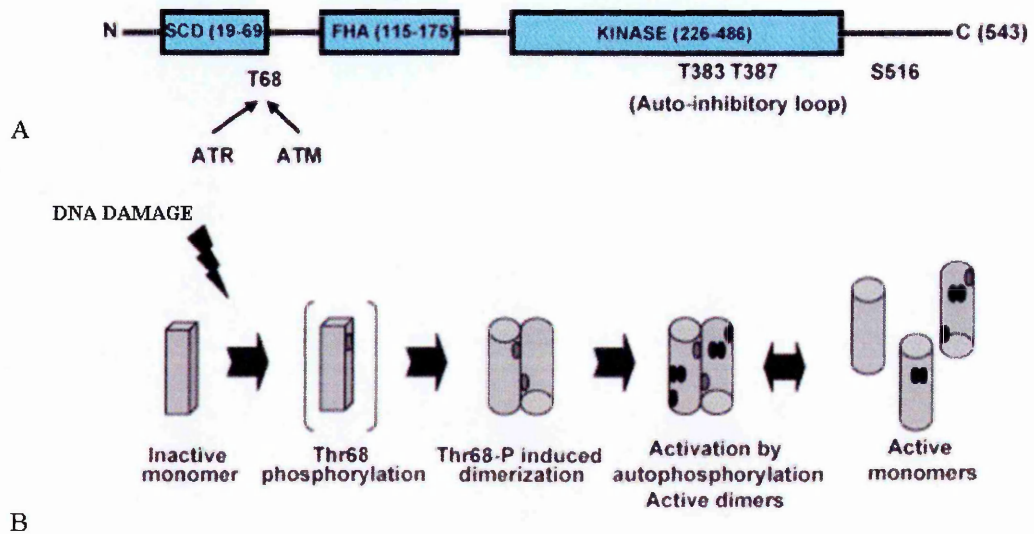


Figure 1.11 Structure and activation of human Chk2

Chk2 functional domains (A) and a model of Chk2 activation following DNA damage (B). (Modified by Ahn J. et al. 2004)

transition as the Cdk1/cyclinB1 can not be activated (Peng et al., 1997; Dalal et al., 1999). Chk2 phosphorylates E2F1 at ser364 in response to DNA damaging agents thus regulating both E2F1 stabilization and transcriptional activity (Stevens et al., 2003) which is strictly related to the regulation of the apoptotic pathway after DNA damage. It has been shown that both BRCA1 and PML can interact with Chk2 and may be phosphorylated by Chk2 following DNA damage (Lee et al., 2000; Yang et al., 2002). In particular Chk2 interacts with both the substrates in the absence of DNA damage and phosphorylates them after DNA damage resulting in subsequent inhibition of binding with Chk2 thus ensuring the activation of other Chk2's substrates. It has been shown that BRCA1 and Nbs1 component of the MRN complex are required for ATM dependent activation of Chk2 after DNA damage (Foray et al., 2003). Many other newly discovered checkpoints mediators are involved in the Chk2 activation pathway such as 53BP1 and MDC1, with mechanisms that have still to be clearly elucidated (Fernandez-Capetillo et al., 2002; Stewart et al., 2003; Hirao et al., 2002).

Differently to Chk1 KO mice, Chk2 deficient mice are viable, fertile and do not show a tumor prone phenotype except when exposed to carcinogens (Hirao et al., 2002; Takai et al., 2002). Although there are some discrepancies in the results among different studies with Chk2 deficient mice and cells derived from them, the observed phenotype is dominated by the increased resistance of the Chk2^{-/-} mice to IR, cellular defects in p53 function and in some checkpoint responses including apoptosis. Recently, in contrast to what was observed in cells lacking Chk1, it was shown that cells lacking Chk2 did not enter mitosis prematurely with incompletely

replicated DNA when DNA synthesis is blocked (Zachos et al., 2005). This is also in contrast to what was observed in budding and fission yeast, where Rad53 and cds1 (Chk2 homologs) are activated when DNA synthesis is inhibited and are considered to be the primary effectors of mitotic delay when replication is blocked (Santocanale and Diffley, 1998; Kim and Huberman, 2001).

It was recently shown that Chk2 can interact with the Polo Like Kinase 1 (PLK1), a centrosome associated kinase implicated in mitotic progression colocalizing with this protein in the centrosomes. Interestingly PLK1 can phosphorylate residues in the N-terminus of Chk2 including Thr68 (Tsvetkov et al., 2003). The related kinase PLK3 also interacts with Chk2 and phosphorylation of Chk2 and PLK3 by one another has been reported (Xie et al., 2002; Bahassi et al., 2002).

1.7.3 CHK1 AND CHK2 INTER-RELATIONSHIP WITH p53 AND p73

The cell cycle checkpoint kinases Chk1 and Chk2 have been shown to phosphorylate multiple sites in the N terminal domain of p53 leading to p53 stabilization and activation (Shieh et al., 2000; Chehab et al., 2000; Meek, 1998). Recently, additional Chk1 and Chk2 phosphorylatable sites have been identified in the C-terminal domain of p53, upon DNA damage (Hirao et al., 2000). These C terminal phosphorylations would differentially regulate C terminal p53 acetylations thus having a role in the differential p53 downstream target genes. Although it is probable that Chk1 and Chk2 are involved in the N terminal phosphorylations *in vivo*, gene KO or ablation by siRNA in certain cancer cell lines questioned this possibility (Ahn et al., 2003; Jallepalli et al., 2003). For example it was shown that the elimination of Chk2 by

gene KO or its downregulation by siRNA resulted in only partial or no defects in the DNA damage induced phosphorylation and stabilization of p53. This is also consistent with the Chk2^{-/-} mice model proposed by Takai et al. where only a 30-50% reduction in p53 stabilization was observed and human p53 introduced in MEFs Chk2^{-/-} resulted phosphorylated normally after IR, indicating that in murine tissue, Chk2 makes a partial contribution to p53 stabilization while other factors clearly contribute (Takai et al., 2002). No G2 or S phase checkpoint was observed in this Chk2 null mice model but they did observe a deficient G1-S arrest consistent with impaired p53 signaling. Moreover the p53 derived peptide containing the Chk2 targeted phosphorylated sites thr18 and ser20 is a poor substrate for Chk2 and do not fit with the consensus sites found in other Chk2 substrates, thus increasing the controversial effective role of Chk2 in phosphorylating p53 *in vivo*.

In fact the N-terminal activation domain of p53 does not contain the classical substrate motif of Chk1 and Chk2 and the physiological tetrameric form of p53 seems to be required for an efficient phosphorylation of thr18 and ser20 of p53 by these two kinases (O'Neill et al., 2002; Seo et al., 2003). Furthermore it turns out that to be efficiently phosphorylated by Chk1 and Chk2, p53 requires physical binding to docking sites in its central domain inducing allosteric changes in Chk1 and Chk2 (Craig et al., 2003).

A strict relationship between Chk1 and p53 has been put forward by two different groups (Damia et al., 2001; Gottifredi et al., 2001), suggesting that upon damage Chk1 phosphorylates and activates p53, that is then able to induce a downregulation of Chk1. This is likely to occur in cells with moderate damage, in which repair of the

lesion has occurred and hence the cell cycle needs to be restarted after the transient block. It has been shown that the p53 dependent repression of Chk1 requires the transactivation function of p53 and is mediated by p21 and pRB (Gottifredi et al., 2001). The strong p53 dependent decrease of Chk1 protein levels at late time points after drug treatments together with a related observation that cell lines with a wt p53 express lower levels of Chk2 compared with mutant p53 expressing cells (Tominaga et al., 1999), can be considered the way the cells tend to abrogate the G2 block induced by anticancer treatments. This phenomenon is consistent with the observation that in cancer cells lacking p53 there is a more prolonged and sustained arrest in the G2 phase of the cell cycle than in cells expressing a functional wild-type p53 (Pollack et al., 1996; Sugiyama et al., 2000). This subtle inter relationship existing between p53 and Chk1 suggests how the different molecular components participating in the cell cycle checkpoint cascade activated upon DNA damage can be closely linked. This is further confirmed by the recent data establishing an inter-relationship among Chk1, Chk2 and p53 related gene p73. It has been shown that Chk1 and Chk2 control the induction of the p53 related transcription factor p73 following DNA damage, through the modulation of the stabilization and activation of E2F1, which is involved in the activation of the TAp73 promoter (Urist et al., 2004). It was previously observed indeed that the E2F1 transcription factor can induce p73 mRNA and p73 is required for E2F1-induced apoptosis (Irwin et al., 2000; Lissy et al., 2000; Stiewe and Putzer, 2000). Consequently the ability of Chk1 and Chk2 to regulate the expression of E2F1 a factor involved in the regulation of TAp73 transcription, is critical for induction of p73 and for their indirect role in apoptosis as well. Furthermore Chk1 but not Chk2 was recently shown to

phosphorylate *in vitro* and *in vivo* p73 at ser47 (Gonzalez et al., 2003), after DNA damage, thus possibly contributing to the p73 α apoptotic response to DNA damage.

1.8 E2F FAMILY IN CELL CYCLE REGULATION AND CHECKPOINTS

The E2F family is a very important group of transcription factors linked with cell cycle control and apoptosis. The founding member E2F1 was discovered in 1980 as a transcriptional activator of the adenovirus E2 promoter (Kovesdi et al., 1986), then the family grew through the addition of new members in mammals and the discovery of homologs in other eukaryotes.

1.8.1 E2F FAMILY MEMBERS ORGANIZATION AND REGULATION

The E2F family consists of eight proteins in mammals: E2F1-6 are the older and better characterized members, while E2F7 and E2F8 have been only recently identified (de Bruin et al., 2003; Maiti et al., 2005), bearing only little homology with the rest of the family members. The established six components (E2F1-6) possess N terminal DNA binding domain and dimerization domains with the marked box, a conserved region involved in dimerization and DNA bending. They all have a C terminal transactivation domain (with the exception of E2F6) containing the pocket protein binding region. The E2F family is divided into three groups based on their different homology. E2F1-3 constitutes the activating group due to their ability to induce S phase entry in quiescent cells and get arrested by the p16ink4a cdk

inhibitor (CDKI) (Johnson et al., 1993; DeGregori et al., 1997; Lukas et al., 1996). On the other hand E2F4 and 5 constitute the repressor group which is important for cell cycle exit and terminal differentiation processes (Fajas et al., 2002). Finally E2F6, has a unique ability to interact with members of the mammalian polycomb complex (PcG) and not with the pocket proteins like the other members do (Morkel et al., 1997; Cartwright et al., 1998). Functional E2F depends on the formation of heterodimers with members of the DP family of transcription factors, which have significant homology with E2Fs sharing the dimerization and DNA binding domains (Girling et al., 1993). The cooperation between E2F and DP is suggested to optimize DNA binding activity in a synergistic fashion (Bandara et al., 1993; Krek et al., 1993). The E2F cell cycle activity is mainly dependent on the phosphorylated state of the pocket proteins. The pocket protein family includes the retinoblastoma protein, pRb, which is the most prominent member, p107 and p130 (Classon and Harlow, 2002; Zhu et al., 1993; Cobrinik et al., 1993). They all share the ability to regulate cell cycle progression and arrest cells in G1 when they are overexpressed (Qin et al., 1992). Interestingly pRb deficient mice die in the embryogenesis while p107 and p130 deficient mice do survive thus not behaving as classical tumor suppressor. Generally when a pocket protein is hypophosphorylated, it can physically associate and inactivate E2F. The pocket protein/E2F-DP complex is guided to E2F binding sites in different promoters where it can recruit histone deacetylases (HDACS) and suppress transcription by remodeling the nucleosome (Ferreira et al., 1998; Magnaghi-Jaulin et al., 1998). The first and most important pocket/E2F interaction detected was that between pRb and E2F1 (Helin et al., 1993). E2F gets activated

when cyclin/CDKs phosphorylates the pocket protein relieving E2F from its inhibitory effect, having next the ability to interact with histone acetyltransferase (HAT), which can act as coactivator (Brehm et al., 1998). E2F1-3 bind selectively to pRb, while E2F5 binds to p130 and E2F4 interacts with all pocket proteins (Dyson, 1998). Other than the pocket protein interaction as E2F regulation, this family present additionally subtle regulator aspects, such as transcriptional control, subcellular localization and post translational modifications. It has been shown that the transcription of activating E2Fs is subjected to an E2F dependent self inhibitory loop regulation (Araki et al., 2003; Adams et al., 2000). CyclinA/CDK2 phosphorylates E2F1 at serine 375 and increases its binding affinity for pRb (Peeper et al., 1995). On the other hand phosphorylation at ser403 and thr433 within the activation domain by the basal TF TFHII triggers E2F degradation (Vandel and Kouzarides, 1999). ATM/ATR dependent phosphorylation of E2F1 induces its activity by inhibiting its degradation through the action of the phosphoserine protein 14-3-3 tau (Wang et al., 2004). Additionally, E2F can be posttranslationally modified by acetylation with probable effect to increase their halfives and DNA binding activity (Marzio et al., 2000; Martinez-Balbas et al., 2000).

1.8.2 E2F FAMILY FUNCTIONS

The E2F family, especially the founding member E2F1, has to be considered as a central component of the cellular machinery that regulates a wide spectrum of genes involved in various functions. In fact a great number of genes implicated in cell cycle control, DNA licensing and synthesis, mitosis DNA repair and apoptosis contains the

E2F responsive site in their promoter ([T/C]TT[C/G][G/C]CG[C/G]) (Slansky et al., 1993). The most studied and best understood function of E2F is its ability to regulate the G1/S transition and S phase entry during the cell cycle. During G0 and early G1, E2F activity is mediated by E2F4 and 5 which are bound preferentially to p130 exerting an inhibitory effect on the E2F responsive genes. At the same time the activator E2Fs are bound and inactivated by pRb. As the cells progress in late G1 phase, pRb and p130 are phosphorylated at first by cyclinD/CDK and later by cyclinE/CDK thus releasing the activating E2Fs (Lundberg and Weinberg, 1998). It's noteworthy to underline that E2F is not only a simple regulator of the G1/S transition transcriptional machinery and this is evidenced by the finding that many putative E2F target genes have functions required for mitosis (DeGregori, 2002). These include genes with functions in centrosomes duplication, spindle checkpoints, chromosome condensation and segregation, cytokinesis and centromeric proteins. Mad2, a known E2F target gene involved in spindle checkpoint appears misexpressed in cells with deregulated E2F activity and this leads to mitotic defects and aneuploidy (Hernando et al., 2004). Experimental evidences attributed to E2F a role in the inhibition of the anaphase promoting complex further confirming its crucial role in cell cycle progression (Garbe et al., 2004). Furthermore it has been shown that E2F3 inactivation leads to centrosomes amplification, mitotic spindle defects and aneuploidy suggesting that E2F3 functions to coordinate the DNA replication with the centrosome duplication cycle (Saavedra et al., 2003). Several experimental evidences suggested a functional role for E2F1 in the DNA damage response. E2F1 protein levels increase in cells treated with DNA damaging agents and

this induction can be attributed to an increase stability of the protein principally due to its phosphorylation at ser31 by ATM and ATR and additionally to the phosphorylation at ser364 by Chk2 upon DNA damage (Stevens et al., 2003). Damage induced E2F1 activity can result in p53 dependent apoptosis through both the expression of proapoptotic p53 cofactors and by inducing the expression of p14. The apoptotic activity of E2F1 can also occur by p53 independent mechanisms through p73, caspase ,9 and the pro apoptotic members of the Bcl2 family (Stiewe and Putzer, 2000; Seelan et al., 2002; Nahle et al., 2002; Hershko and Ginsberg, 2004). Interestingly the p73 promoter region contains at least three E2F1 binding sites and the enforced expression of E2F1 strongly stimulates the transcription of p73 through the direct binding to the E2F1 responsive elements in the p73 promoter (Irwin et al., 2000; Stiewe and Putzer, 2000). The E2F1 mediated regulation of p73 results in a significant induction of apoptosis. E2F1 acetylation is required to induce p73 transcriptional activation (Pediconi et al., 2003).

Several studies had also suggested a role for E2F in checkpoint control. Upon DNA damage and ATM phosphorylation, the transcriptional activity, the apoptotic and the S phase inducing activities of E2F1 are inhibited by binding to TopBP1 which recruits E2F1 to BRCA1 containing nuclear foci. Furthermore E2F1 has been implicated in the recruitment of the Mre11 recombination repair complex to origins of DNA replication, suggesting a direct role for E2F1 in DNA damage checkpoint or repair (Liu et al., 2003).

1.9 DNA DAMAGE CHECKPOINT PATHWAYS AND CANCER THERAPY

Current approach in cancer therapy to obtain more encouraging clinical response, focuses on the identification of specific molecular targets against which drugs can be developed. Cancer is a complex disease caused by many genetic abnormalities, so interfering with a single target might not be sufficient to produce a response. On the other hand many tumors share similar characteristics such as genetic instability and high proliferative rates which can be targeted by various DNA damaging agents.

Anticancer agents targeting DNA are the most effective in clinical use and have produced significant increases in the survival of patients with cancer when used in combination with drugs that have different mechanisms of actions. These agents can be divided into four main classes: alkylating agents (such as cis-platinum), anti metabolites (such as 5-FU), topoisomerase inhibitors (such as etoposide) and radiomimetics (such as bleomycin). Despite their effectiveness as anticancer drugs, these agents have some key limitations. In fact many patients with cancer either do not respond or develop resistance to them. These agents can also be extremely toxic and show only a very limited therapeutic window, also known as therapeutic index representing the ratio between the toxic dose and the therapeutic dose of a drug. The selectivity of these treatments depends on quantitative differences in the rates of division between cancer and normal cells and on the presence of tumor defects in pathways that are related to the target of the treatment such as particular checkpoint signaling pathways. A good strategy to improve the therapeutic window would be either to reduce the activity of the checkpoint and DNA repair in tumor cells or to

reduce the damage induced apoptosis in normal cells (Zhou and Bartek, 2004). Among the genes and pathway commonly altered in human cancer, p53 represents the best example, being compromised in over 50% of human tumors thus representing the most frequently altered gene in humans. This can be due to p53 mutations, overexpression of its negative regulator mdm2 or inactivation by the E6 of an oncogenic human papillomavirus (HPV) (Hollstein et al., 1991; Michael and Oren, 2002). p53 deficiency alters the cellular response to DNA damage leaving the cells with attenuated DNA damage checkpoint controls and a reduced propensity to undergo apoptotic cell death. Thus the DNA repair capacity of these cells is reduced but the survival is increased, promoting genomic instability and contributing to the resistance of the p53 deficient cells to cytotoxic agents. As p53 has a crucial role in the G1 checkpoint, resistance of p53 deficient cancer cells to therapy is at least in part due to their ability to repair DNA damage during G2 phase arrest. A good strategy to sensitize specifically those p53 mutated cells to anticancer agents would be to disable the G2 checkpoint favoring the accumulation of DNA damage and the subsequent death of the only cancer cells while the normal cells with an intact p53 and G1 checkpoint would not be affected by such sensitization. Recently many efforts have been put in studying the signal transduction pathways that mediate the responses to various DNA damaging agents and in particular the two G2 checkpoint kinases Chk1 and Chk2 as possible key target molecules whose inhibition will ultimately lead to a new generation of adjuvant therapeutics which could significantly improve the efficacy and selectivity of DNA damaging agents in the clinic (Dixon and Norbury, 2002).

1.10 AIMS

The DNA damage response is essential to ensure cellular life and to avoid tumorigenesis. It occurs by rapidly transducing the damage signal to many intracellular systems such as the DNA repair mechanism and the cell cycle checkpoints. As outlined in the introduction (chapter 1.2-1.6), the cell cycle checkpoint consists of regulatory mechanisms that do not allow the initiation of a new cell cycle phase before the previous one is completed and temporarily arrest the cell cycle whenever damage is sensed to allow time for DNA repair or to promote apoptosis if the damage is unreparable. The misfunction of these checkpoints promotes genetic instability favoring neoplastic transformation. In the majority of cancer cells one or more of DNA damage checkpoints components have lost their function. The better understanding of the role of these checkpoint proteins in tumor cells may represent a starting point for the design of new molecules against cancer targets to be used in cancer therapy in combination with common anticancer agents with the aim to increase the therapeutic index.

The serine threonine kinase Chk1 was chosen in this project to better define the molecular mechanisms regulating the G2 checkpoint induced after DNA damage. Indeed, recent experimental evidence attributes to this protein a fundamental role in activating not only the G2-M but also the S phase checkpoint following DNA damage. In comparison the oncosuppressor p53, which has been found mutated in a high percentage of human tumors, has a relevant role in the G1 checkpoint (chapter 1.3). The definition of the molecular mechanisms at the basis of the G2 checkpoint assumes particular importance in tumor cells, as cells almost invariably harbor defects in the G1 checkpoint, due to p53 mutation, but experience a persistent G2

block after anticancer drug treatment. One might hypothesize that the abrogation of this residual G2 checkpoint by inhibiting specific targets renders tumor cells more sensitive to anticancer treatments than normal cells having an intact G1 checkpoint.

The rationale of the work described in this thesis is three-fold:

- I. The starting point of the first part of my thesis relies on the previous observation of the existence of an interplay between Chk1 and p53 following DNA damage. Chk1 can contribute to the rapid phosphorylation and activation of p53 soon after the DNA damage. At later time points from the introduction of the damage Chk1 would be downregulated by p53 to try to resume the cell cycle once the damage has been repaired (figure 3.1). In order to further investigate the p53-dependent downregulation of Chk1 and to understand how Chk1 is transcriptionally regulated, the human Chk1 gene promoter has been isolated and structurally and functionally characterized.
- II. The second part of this work describes different strategies attempted to inhibit the Chk1 function in isogenic cellular systems either with an intact G1 checkpoint or with G1 checkpoint defects due to p53 or p21 inactivation with the aim to abrogate the G2 checkpoint following different anticancer treatments and to evaluate the possibly increased sensitivity to such treatments. The colon carcinoma cell line HCT-116 wt and its isogenic systems p53^{-/-} and p21^{-/-} were chosen. The siRNA strategy constituted the most successful technique to inhibit the Chk1 function. It was also used to inhibit in parallel another key molecular player of the G2 checkpoint, the protein kinase Chk2.

III. The third part of my thesis has the goal to further investigate and clarify the physiological role played by Chk1 in different human somatic cell lines in the absence of genotoxic agents.

Taken together the studies described in my thesis have the overall aim to discover molecular mechanisms regulating the G2 checkpoint after DNA damage and to define the possible role played by the kinase protein Chk1 as a potential target for anticancer therapy.

CHAPTER 2

MATERIALS AND METHODS

2.1 CELL CULTURE

2.1.1 CELL CULTURE CONDITIONS

Cell culture procedures were carried out aseptically in a class II laminar flow hoods. Cells were maintained in a Heraeus CO₂ Auto-Zero incubator at 37°C with 5% CO₂ and tested for mycoplasma contamination once a month. The human cancer cell lines used in my investigations were: the ovarian cancer cell lines SKOV-3 and A2780, the osteosarcoma cell lines SAOS-2 and U2OS, the colon carcinoma cell line HCT-116 and the cervical cancer cell line HeLa with the specific human papillomavirus type 16 E6 gene integrated in the genome (coding for the E6 protein which binds to p53 and targets it for accelerated ubiquitin-mediated degradation). They were all obtained from The American Type Culture Collection (ATCC, Rockville, MD, USA). The two human ovarian carcinoma cell lines were maintained in RPMI-1640 media supplemented with 10% fetal calf serum and 2 mM L-Glutamine. The osteosarcoma cell lines and HeLa cells were maintained in DMEM supplemented with 10% of FCS and 2 mM of L-Glutamine. The HCT116 human colon-carcinoma cell line was maintained in ISCOVE's modified medium supplemented with 10% fetal calf serum and 2 mM L-Glutamine.

The SAOS-2-E2F1 clone was previously obtained in our laboratory after stable transfection and selection in G418 (500µg/ml) of an expression plasmid encoding for a wt hE2F1, kindly provided by Dr. Helin, (European Institute of Oncology, Milan, Italy). Retinoblastoma (Rb) inducible H562 human small cell lung cancer cells (clone 10D5) were kindly provided by Dr. G.W. Kristal (Mc Guire VA, Medical Center 111K,

Richmond, Virginia) and were grown in RPMI 1460 supplemented with TET System Approved FBS (fetal bovine serum) (BD Palo Alto, US) which replaced the fetal calf serum. The HCT-116 tetracycline inducible clones used in my work were maintained in the same medium as the parental cell line, supplemented with TET System Approved FBS as well as the Rb inducible clone. The tetracycline inducible clones derived from the human carcinoma cell line HCT116 and HCT116 p53 $-/-$, clone Trex 8A and clone Trex15A, were previously isolated in my laboratory. These clones were maintained under selection with blasticidine (working solution: 5 μ g/ml). The clones derived from the human colon-carcinoma cell line HCT-116, clone 379.2 originated by targeted deletion of the p53 gene (p53 $-/-$) and clone p21 $-/-$ originated by targeted deletion of the p21 gene (p21 $-/-$) were kindly supplied by Dr. Vogelstein from John Hopkins University (Baltimore, MA, USA).

The different media were purchased from Sigma and contained the mineral and supplements necessary for the growth of the cells except for the serum, which was added when needed. Cells were passaged routinely before they reached confluence, to maintain a logarithmic growth. The cells were replaced with fresh liquid nitrogen stocks after every four-five months of culture. Procedures to detach and subculture cells were the same for all the cell lines used and consisted of two washes with warm sterile phosphate buffer saline (PBS, Sigma) and detachment with a solution of 1X trypsin/EDTA (Sigma). The trypsin activity was stopped by adding calf serum-containing medium. After centrifugation at 1,200 rpm for 10 minutes, cells were resuspended in the appropriate medium counted by the Counter method (Coulter Counter, ZM) and seeded at the desired density.

2.1.2 LONG TERM STORAGE OF CELLS

To generate and maintain batches of cells, exponentially growing cells were washed twice with PBS and centrifuged at 1,200 rpm for 10 minutes at room temperature. The cell pellet was resuspended in culture medium containing 50% of cryoprotective medium (Bio-Whittaker, Milan-Italy) and 20% of calf serum to a density of 5,000,000 cells/ml. Aliquots of 1 ml were kept on ice for 30 minutes, cooled slowly for 3 h in liquid nitrogen vapour and then immersed in liquid nitrogen. Cells were recovered from the cell bank by rapid thawing to 37 °C in a water bath, centrifuged at 1,200 rpm for 10 minutes, resuspended in the appropriate culture medium and transferred to a tissue culture flask. The day after, the medium was removed and new, fresh medium was added.

2.2 CELL GROWTH ANALYSIS

2.2.1 PREPARATION OF DRUG SOLUTIONS

Cis-dichloro-diamminoplatinum (DDP) was prepared freshly for any experiment by preparing a solution of 0.5 mg/ml in medium and allowing this solution to equilibrate with proteins present in the serum at 37°C for 30 minutes before treatments. Etoposide (VP-16) was prepared in DMSO as 5mM stock solution and subsequently diluted in fresh medium the day of treatment. The stock solution was maintained at -20 °C. Hydroxyurea (HU) was made freshly for each experiment by preparing a solution 500 mM concentrated in medium with no serum. The dissolution was enhanced by vortexing

for few seconds. IR treatments were made using the RadGil Machine (Gilardoni-Italy), while a UV lamp was used to UV irradiate the cells.

2.2.2 CELL GROWTH CURVE

To detect the effects on the cell growth of the small interfering RNA transfected cells (in HCT116 p53 +/+, p53 -/- and p21 -/- and in U2OS cells), of the cells inducible expressing the dead kinase Chk1 and of the single or double inducible expressing Chk1 siRNA, the growth of the cells was followed by counting the cells through the Counter Method (Coulter Counter, ZM) each 24 hours after transfection or induction with the inducer doxycycline (2µg/ml) (a more stable tetracycline analogue), starting from 24 hours and ending up to 144 hours after transfection. This investigation thus permitted the construction of the cell growth curve.

2.2.3 COLONY ASSAY

The effects of DDP and IR on mock or siRNAs transfected cells (HCT116 and isogenic cell lines), the effects of DDP and UV on the doxycycline inducible HCT-116 clone overexpressing the dead kinase Chk1 and the effects of DDP and VP-16 on the HCT116 cell lines induced to express the single siRNA Chk1 were evaluated by a standard clonogenic assay. Briefly, in the first case, cells plated on 24 well plates were treated, 48 hours after transfection with siRNAs, with different doses of DDP for two hours or irradiated for different time. At the end of each treatment, cells were washed in PBS, trypsinized and seeded at 200 cells/ml (HCT 116 and HCT 116 p53-/- cells lines) and

400 cells/ml (HCT 116 p21^{-/-} cell line) in 6 well plates. Colonies were allowed to grow for about 8 to 10 days. When the control cell colonies were visible under the microscope, the medium was aspirated and the wells washed with 1 ml of PBS. Then the colonies were stained with crystal violet (BDH, Milan, Italy) by adding 1 ml of the commercially available solution (1% concentrated) and, after extensive washes in water, with the final two washes in distilled water, the plates were air dried and the number of stained colonies counted by the Entry level Image System (Immagini & Computer, Italy). A background correction was made and the smallest control colony was taken as the minimum for the establishment of the cut-off point. All the data are the mean \pm SD of at least three experiments, done in triplicate.

In the second and third case, cells were seeded at low density in 6 well plates and either induced or not with doxycycline 2 μ g/ml every 48 hours. At 72 hours from the first induction cells were treated with different doses of drugs for 2 hours. At the end of each treatment, cells were washed in PBS, recovered in the proper culture medium and stored at 37 °C in the incubator leading to the formation of the colonies. The subsequent steps of staining and data analysis were performed as above described.

2.3 GENOMIC DNA ANALYSIS

2.3.1 SCREENING OF GENOMIC LIBRARIES

Genomic clones containing the genomic Chk1 sequences were isolated by screening a genomic library spotted on filters obtained through the UK Human Genome Mapping Project Resource Centre (UK-HGMP-RC). The human PAC library RPCII has been

constructed in the vector, pCYPAC2N. The source is a normal male blood donor, and the insert size is about 110 kb. The library consists of approximately 120,000 clones which have been spotted on 22.2 x 22.2 cm Hybond N nylon membranes (Amersham). Each clone has been spotted twice to give 36,864 (18,432 x 2) spots on each membrane. Seven filters cover the whole library. The 7 filters were prehybridized at 65°C in 25 ml of a solution containing 6x SSC, 100 µg/ml of salmon sperm, 0.5% SDS and 5x Denhardt's solution (a 50x Denhardt solution is prepared by dissolving 1 g of Ficoll 400, 1 g of polyvinylpyrrolidone and 1 g of BSA fraction V in 100 ml of water; the solution is then filtered and stored at -20°C) with gentle, continuous agitation. To this solution, radiolabeled specific Chk1 cDNA probe containing a 5' Chk1 genomic region of 170 bp (prepared as reported next in paragraph 2.3.2.5) was added and the incubation continued for further 16 hours at 65°C. The filters were then washed twice at room temperature in 2x SSC, followed by two washes of 15 min each at 65°C with 2 x SSC + 1% SDS and a final wash in 2x SSC without SDS for 10 minutes and exposed to autoradiographic films. Once developed, the films were oriented with the grid supplied with the filters and the positive clones identified. This procedure was facilitated by the double spotting of each clone.

The 2 identified positive clones, called 53p13 and 253, were requested to the UK-HGMP-RC and obtained as glycerol stock. From these stocks, the PAC clones were isolated using the Qiagen midi preparation kit following the procedures reported in the instruction manual.

2.3.2 SOUTHERN BLOT ANALYSIS

Briefly, this technique consists in transferring DNA molecules from an agarose gel onto a nitrocellulose membrane. Southern blotting is designed to locate a particular sequence of DNA within a complex mixture. In our specific investigation this approach was used to isolate shorter genomic fragments containing the 5' region of the Chk1 gene from the isolated DNA of the PAC clones.

2.3.2.1 DNA RESTRICTION ENDONUCLEASE DIGESTION

Before loading onto an agarose gel, genomic DNA must be digested with one or more restriction endonuclease enzymes. An aliquot of the genomic DNA previously isolated (usually around 20µg) was digested with different restriction enzymes in their appropriate buffer supplied together with the enzyme, for 16 hours at 37°C. The total volume of the digestion is 100 µl containing 10 µl of appropriate 1X dilution buffer and 5 µl of restriction enzyme.

2.3.2.2 DNA ELECTROPHORESIS

The entire restriction reaction was loaded on 0.8% agarose gels before adding 1/6 of volume of loading buffer. After loading, the gel electrophoresis was performed in TAE buffer (1X) at a constant voltage of 80 V, for 5-6 hours.

Agarose gel is prepared by dissolving 1g of ULTRA pure agarose (Sigma) in 100 ml of 1X TAE buffer:

TAE Buffer (50X)

final concentration

242 g Tris base 2 M

100 ml EDTA 0.5M, pH 8 50 mM

57.1 ml glacial acetic acid

to 1 l with deionised water.

The solution was stored at room temperature.

The solution was heated in a microwave oven and boiled until all the agarose is dissolved. The solution was then cooled to approximately 50°C before pouring in a casting tray. At the end of the run the gel was stained in a solution of 1 µg/ml of ethidium bromide in TAE buffer for 30 minutes and the DNA bands visualized with a UV transilluminator to visualize a smear which is indicative of a good digestion and separation. The DNA was then denaturated into single stranded form before blotting onto a membrane.

2.3.2.3 DNA DENATURATION

The agarose gel was placed in a box containing 250 ml of denaturation solution and shaken gently for 45 minutes.

| <i>Denaturation solution</i> | <i>final concentration</i> |
|------------------------------|----------------------------|
|------------------------------|----------------------------|

| | |
|---------------|-------|
| 30 ml NaCl 5M | 0.6 M |
|---------------|-------|

| | |
|---------------|-------|
| 20 ml NaOH 5M | 0.4 M |
|---------------|-------|

to a final volume of 250 ml with deionised water

After this incubation, the agarose gel was rapidly washed in deionised water and incubated for 45 minutes in neutralization solution.

| <i>Neutralization solution</i> | <i>final concentration</i> |
|----------------------------------|----------------------------|
| <i>125 ml Tris-HCl 1M pH 7.5</i> | <i>0.5 M</i> |
| <i>75 ml NaCl 5M</i> | <i>1.5 M</i> |

to a final volume of 250 ml with deionised water

Before to proceeding with blotting, the agarose gel was washed with deionised water.

2.3.2.4 BLOTTING PROCEDURE

A capillary blot was set up using a solution of 20X SSC as transfer buffer.

| <i>SSC Buffer (20X)</i> | <i>final concentration</i> |
|--------------------------|----------------------------|
| <i>175.3 g NaCl</i> | <i>3 M</i> |
| <i>88.2 g Na citrate</i> | <i>0.35 M</i> |

to 1 l with deionised water.

The pH was adjusted to 7 with NaOH and the solution autoclaved.

The wick was made up of 2 pieces of 3MM paper with each end dipping into the transfer solution. The gel was placed on the wick with wells facing down, and air bubbles were gently removed. A nylon membrane (GeneScreen plus, NEN) of exactly the same size of the gel was soaked in 20X SSC for at least 10 minutes and carefully placed on top of the gel and gently squeezed to remove air bubbles. Three sheets of 3MM paper where then added over the membrane followed by Kleenex towels. The blot system was left for 16-24 hours after which the membrane was removed, washed by immersion in deionised

water, left to dry at room temperature for approximately 30 min. The blot was stored at 4°C until ready for hybridization.

2.3.2.5 PREPARATION OF PROBE FOR SOUTHERN BLOT ANALYSIS

The 5' Chk1 gene specific probe of 170 bp was the same used also for the screening of the genomic libraries and was prepared by using the gene insert excised from the plasmid by restriction enzyme digestion. The radioactive probe was prepared using a Klenow fragment of polymerase I to copy the probe sequence while incorporating radioactive nucleotides. A commercially available kit (Rediprime, Amersham) was used for this purpose. For labelling, 50 ng of DNA as starting material were used and all the steps described in the manual were followed using as radioactive nucleotide ³²P-labeled dCTP (Amersham, 3,000 Ci/mmol). Purification of the probe from unlabelled nucleotide was performed using Sephadex G50 columns (Boehringer-Mannheim) exactly following the manufacturer's instructions. The incorporated radioactivity was detected by Cherenkov counting and the probe was diluted to have a final concentration of 500,000 cpm/ml of hybridization solution. Hybridization and washing conditions were the same used for the screening of the genomic filters. The autoradiograph was compared to the ethidium bromide picture and the bands which hybridized with the probe identified. A second digestion was then performed on the remaining genomic DNA maintaining exactly the same conditions previously used.

Once separated on the agarose gel, the band chosen of about 10 kb was excised and isolated using the QIAgel extraction kit (Qiagen) and cloned in a pBluescript plasmid (BSSK) following the procedure I will describe in the next paragraph.

2.4 MOLECULAR CLONING STEPS AND GENERATION OF CELLULAR CLONES

2.4.1 PREPARATION OF CONSTRUCTS

During the work I'm presenting in this thesis I applied the molecular cloning strategy several times. To transfer the gene of interest in the genome of host cells by a specific vector, I used, depending on the necessity, two possible strategies: 1) excising the fragment from an existing construct; 2) cloning the fragment by PCR. In the first case the DNA was excised from the plasmid of origin by restriction endonuclease digestion and subcloned in the appropriate expression vector. The expression vectors used in our experiments were the pCDNA vectors series produced by Invitrogen for stable or transient overexpression of the cDNA of interest (pCDNA3 vector represented in figure 2.1). The principal features of these plasmids are: the presence of a promoter driving the transcription, a polylinker sequence containing the DNA recognition sequence for many restriction enzymes to facilitate the subcloning of the gene of interest, also called MCS (multiple cloning site), a bacterial resistance gene (ampicillin) for selection of recombinants and an eukaryotic resistance gene (neomycin, blasticidin, zeocin or puromycin) for selection of cells containing the plasmid. To test the promoter activity of Chk1, the different genomic fragments were subcloned in the pGL2 enhancer vector lacking the promoter and with the MCS upstream to the reporter gene luciferase. To subclone the gene of interest, the general molecular biology techniques reported in Sambrook et al. (1989) were used. In summary, the gene of interest is excised from the plasmid of origin by digestion with the appropriate restriction enzymes for 1 hour at

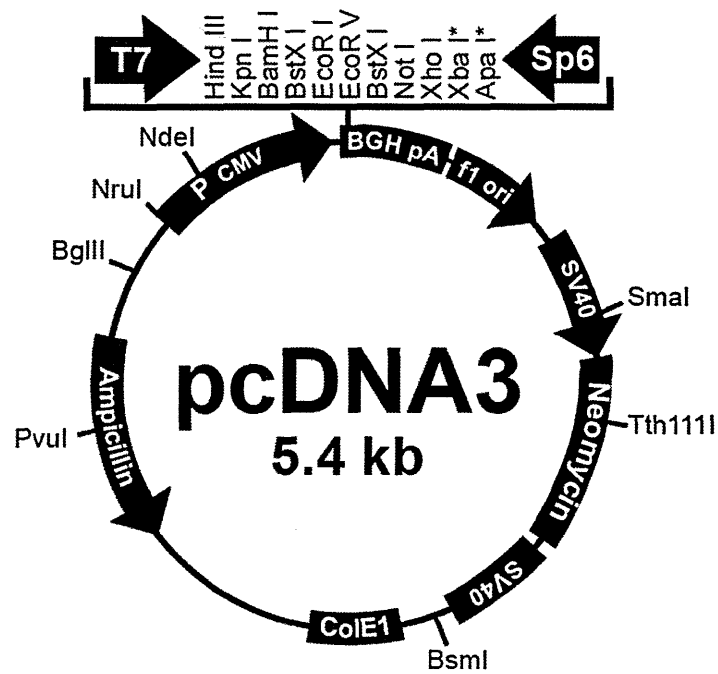


Figure 2.1 pcDNA3 expression vector

Circular plasmid for stable or transient over-expression of the cDNA of interest in eukaryotic cells.

37°C in a buffer supplied with the enzyme. The entire reaction is loaded onto a 1% agarose gel to separate the exogenous DNA fragment from the plasmid by electrophoresis (gel preparation as described in paragraph 2.3.2.2). When the expected bands were present, the appropriate band containing the cDNA was excised from the gel and the DNA extracted from the agarose slice using the QIAgel kit (Qiagen) exactly following the manufacturer's instruction. The cDNA so obtained was stored at 4°C. The host plasmid was digested with the same restriction enzymes used to excise the cDNA from the original plasmid. In case of restriction digestion with a single enzyme, after digestion, the plasmid was treated with shrimp alkaline phosphatase at 37°C for 60 minutes to remove the 5' phosphates and to reduce the probability of the plasmid to recirculate without insert. The reaction was then heated at 70 °C to inactivate the phosphatase. The linearized host plasmid was ligated with the excised cDNA insert in 20 microliters of a solution containing 1U of T4 DNA ligase (Promega) and 1X ligase buffer (30 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 10 mM DTT and 1 mM ATP, supplied with the ligase) for 4 hours at room temperature. Half of the volume of this solution was then used to transform competent bacteria. The second possibility of cloning a sequence of DNA was to amplify by PCR the target sequence and to use the TA strategy to clone the amplified fragment. This technique provides a one step cloning strategy for a direct insertion of PCR product into a vector (T-vector). Basically, Taq enzyme has a non-template dependent activity, which adds a set of deoxyadenosines to the 3' ends of PCR products. The T-vector contains in its multi cloning site (MCS) complementary unpaired 3' thymidyl residues. This allows the PCR products to ligate efficiently with the vector. PCR products were ligated to the vector at a ratio of 1:1 or 3:1 following the

manufacturer's instructions. pGEM[®]-T easy vector was purchased ready made as a component of a cloning kit (Promega, Milan-Italy).

In my work this second possibility was applied to generate the two recombinant Chk1 KO construct. As I will further describe in paragraph 2.8 of this chapter to clone the 3' and 5' arms of the constructs I had to amplify definite genomic regions by PCR strategy using as a template the 53p13 PAC clone, 5' Chk1 gene positive, previously isolated after the screening of a genomic library.

2.4.2 PREPARATION OF COMPETENT BACTERIAL CELLS FOR TRANSFORMATION

Fifty ml of sterile bacterial cellular suspension (XL1-blue, Stratagene) were mixed, in a sterile 50 ml conical tube, with 10 ml of sterile LB medium:

| <i>Luria-Bertani Broth (LB)</i> | <u><i>final concentration</i></u> |
|---------------------------------|-----------------------------------|
| <i>10 g bacto-tryptone</i> | <i>1% (w/v)</i> |
| <i>5 g bacto-yeast extract</i> | <i>0.5% (w/v)</i> |
| <i>10 g NaCl</i> | <i>1% (w/v)</i> |

to a final volume of 1 l with deionised water. The medium was then autoclaved for 15 min and the antibiotic of selection (for pcDNA3 derived plasmids, ampicillin at the final concentration of 50µg/ml) was added once it had cooled to 55°C.

and allowed to grow in a 37°C heated shaking incubator (Folabo) at 225 rpm overnight. One ml of such liquid bacterial culture was then transferred into a sterile 500 ml bottle containing 100 ml of sterile LB medium and the bottle was placed into the 37°C heated

shaking incubator at 225 rpm. To harvest bacterial cells in logarithmic growth phase, 2 hours later, 1 ml of liquid culture was transferred, under a laminar flow in a disposable cuvette and the absorbance at 600 nm wave-length was read on the spectrophotometer. *E. coli* concentration in the liquid culture was calculated by considering that 1 $A_{600\text{ nm}}$ unit corresponds to about 8×10^8 bacterial cells/ml. When the 600 nm absorbance reached $A_{600\text{ nm}} = 0.3$ units (corresponding to roughly 2.4×10^8 cells/ml) the bacterial suspension was transferred in two ice-cold sterile 50 ml conical tubes and the cell growth was stopped by placing the tubes on ice for 15 minutes. *E. coli* cells were pelleted by centrifugation at 3,000 rpm for 10 minutes at 4°C and, after the removal of LB medium, cell pellets were pooled in the same tube by gentle resuspension in 10 ml of ice-cold sterile 0.1 M CaCl_2 solution. After addition of 40 ml of ice-cold sterile 0.1 M CaCl_2 solution, bacterial suspension was incubated on ice for 30 minutes and subsequently centrifuged at 3,000 rpm for 10 minutes at 4°C. The supernatant was then removed and the cell pellet was carefully resuspended in 5 ml of an ice-cold sterile 0.1 M CaCl_2 containing 15% (v/v) glycerol (Sigma). This bacterial suspension was dispensed in 1.5 ml eppendorf tubes (400-500 μl aliquots for each tube) and kept at 4°C for 24 hours after which the tubes were quickly frozen in liquid nitrogen and stored at -80°C . The cells maintained their competence for transfection for 1-2 months when kept at -80°C . Fifty μl of freshly prepared competent cells were transformed with 50 ng of a DNA vector able to confer ampicillin resistance and $1/100,000$ (dilution factor, 10^5) of the bacterial suspension was plated as described in agar plates. Transformation efficiency, calculated on the basis of the formula $(\text{NUMBER OF COLONIES}) \times (10^3) \times (\text{DILUTION FACTOR}) / 50\text{ ng}$, was expressed as the number of colony forming units

(CFU) per μg of plasmide DNA. Generally, 10^6 - 10^8 CFU per μg of plasmid DNA were indicative of a good preparation of competent bacterial cells.

2.4.3 TRANSFORMATION OF BACTERIA

In an ice-cold 10 ml Falcon tube (Falcon, Becton Dickinson), 50 microliters of competent bacterial cells were diluted to a final volume of 100 μl with 0.1 M CaCl_2 and 20 microliters of the ligation reaction (reported in paragraph 2.4.1) were then added. The mixture was gently mixed by tapping and the tube was chilled on ice for 30-40 minutes, incubated for 90 seconds at 42°C in a water bath and for 2 minutes again on ice. After a 5 minute incubation at room temperature, 900 μl of LB medium were added and tube was placed into a 37°C heated shaking incubator at 225 rpm for 1 hour. The tube was then centrifuged at 3,000 rpm for 5 minutes at room temperature, most of the supernatant LB removed and the bacterial cell pellet resuspended in the remaining fluid and spreaded into 90-mm dish (Corning-Costar Italia-Milan, Italy) containing LB agar medium plus the antibiotic of selection: *(LB medium was prepared as previously described. Agar (1.5% w/v) (Life Technologies) was added before autoclaving and the solution poured on 90-mm dish (Corning-Costar, Milan-Italy) and allowed to dry under sterile hood)* and incubated overnight at 37°C without agitation.

2.4.4 IDENTIFICATION OF RECOMBINANT CLONES

Plasmidic DNA was purified from bacterial suspension using the Quiagen mini-prep kit (Quiagen, Milan-Italy). The procedure, starting from 1.5 ml of bacterial suspension was

exactly as described by the manufacturer. The recovered DNA, in 50 μ l of water, was subjected to restriction digestion with appropriate restriction enzymes to verify that the insert was indeed present in the colony isolated. After digestion and separation on agarose gel, the fragments of DNA were visualised by using an UV transilluminator. The positive colonies containing the right insert were stored at -80°C after the addition of 0.5 ml of 50% sterile ultra pure glycerol (Sigma) to 0.5 ml of the bacterial suspension used for the enzyme digestion. When necessary, the remaining DNA was used for confirmation by DNA sequencing, which was performed through custom sequencing services (through a core facility available at Mario Negri Bergamo, Bergamo, Italy and more recently through PRIMM facility, Milan, Italy).

To obtain sufficient DNA to transfect cells, a midi prep purification was set up. Using a sterile spatula, frozen bacterial cells were scraped from the 2 ml sterile Nalgene tube and dissolved in 10 ml of 50 $\mu\text{g/ml}$ ampicillin containing LB medium (contained in a 50 ml conical tube), and allowed to grow at 37°C in a shaking incubator at 220 rpm. After 8 h, 1 ml of liquid culture was mixed with 100 ml of a fresh solution of 50 $\mu\text{g/ml}$ ampicillin in LB medium in a 250 ml glass bottle, and cells were left to grow o/n at 37°C and 220 rpm. Bacterial cells were then pelleted by a 30 min centrifugation at $1,700\times g$ at 4°C . After removal of LB medium, plasmid DNA was purified with the Qiagen Plasmid Midi Kit (Qiagen) according to the manufacturer's instructions. The DNA recovered from the midi preparation was quantified using a spectrophotometer, by reading the absorbance at 260 nm and 280 nm. The quality of the DNA prepared was determined by the ratio between 260 nm and 280 nm absorbances, which should be 1.8.

For quantification the extinction coefficient was used. The amount of DNA was calculated considering that a solution of 50 micrograms of DNA/ml would give an absorbance reading of 1.0 at 260 nm.

2.4.5 TRANSFECTION OF DNA IN EUKARYOTIC CELLS

To transfect DNA into the different cell lines two different techniques were performed: the calcium phosphate precipitate method and liposome method.

2.4.5.1 CALCIUM PHOSPHATE PRECIPITATE METHOD

One day prior the transfection, cells were plated in order to be logarithmically growing on the day of transfection (i.e. 50-60% confluent at the time of transfection). Before transfection, exponentially growing cells were washed twice with ice-cold sterile PBS, and allowed to grow in 4.5 ml of D-MEM medium supplemented with 10% FCS and 2 mM glutamine. DNA was dissolved in sterile water and was mixed with a solution of 2 M CaCl_2 (*prepared by dissolving 14.7 g of CaCl_2 in 100 ml H_2O and filtering the solution with a syringe equipped with a 0.2 μm filter*) to give a final concentration of 0.25 M CaCl_2 in a volume of 250 μl . In the meantime, for each transfection to be performed a 4 ml transparent tube (Falcon) was prepared by adding 250 μl of a 2X HEPES-Buffered Saline (HEBS 2X):

| <i>HEBS 2X</i> | <i>final concentration</i> |
|-------------------|----------------------------|
| <i>1.6g NaCl</i> | <i>280 mM</i> |
| <i>0.074g KCl</i> | <i>10 mM</i> |

*0.027g Na₂HPO₄*H₂O* *1.5 mM*

0.02g Dextrose *12 mM*

1g HEPES *50 mM*

The salts are dissolved in 50-70 ml of distilled water and the pH of the solution is then adjusted to 7.2 with 0.5 N NaOH and then brought to 100 ml with sterile water.

Then a 1 ml sterile pipette fixed to an automatic pipettor was placed at the bottom of the tube containing the HEBS solution and the air forced inside the tube. While the solution was bubbling, the DNA-CaCl₂ solution was added drop-wise. When the DNA has been added, the tube was vortexed for 20 seconds and kept in a tube holder for 30 minutes. The solution was then added drop wise to the cells seeded in a 25 cm² flask and incubated 16 hours at 37°C.

The cells with the DNA-CaPO₄ solution were then visualized under the microscope to verify the presence of small precipitates of Ca₂(HPO₄) on all the surface of the flask. Once this was verified, the medium was removed and the cells extensively washed with PBS (three four times, with the PBS left on the cells for 5 minutes to help in remove as much of the crystals as possible). After washing, the cells were incubated in complete medium.

2.4.5.2 LIPOSOME METHOD

The Lipofectamine 2000 reagent (Invitrogen) was mostly used to transfect DNA by liposome method and the manufacturer's instructions were followed. Briefly, the ratio of DNA (µg) / lipofectamine 2000 (µl) in the transfection mixture prepared were comprised between 1:2 and 1:3 for most of the cell lines utilized. Cells were seeded at

high density one day before transfection in order to have a 90-95% confluence at the time of transfection. Both DNA and liposomes were diluted in Opti-MEM I Reduced Serum Medium (Invitrogen) without serum. After 5 minutes of incubation of Lipofectamine at room temperature, the diluted DNA was mixed with the diluted liposome solution and incubated for 20 minutes at room temperature. The mixture was then added to the cells which were incubated at 37 °C in the incubator till the day of the cells harvesting.

2.4.6 ISOLATION OF CELL CLONES STABLY EXPRESSING THE GENE OF INTEREST

When clones of cells stably expressing the gene of interest had to be isolated, 48 hours after the end of transfection the medium was removed and the cells detached with trypsin/EDTA solution. After counting, the cells were seeded in 9 mm plates at a density of 5,000 cells/ml in medium containing the selection antibiotic (500 micrograms/ml of G418 for neomycin resistance gene containing plasmids, 10 micrograms/ml of zeocin for zeocine resistance gene containing plasmids, 5 micrograms/ml of blasticidin for blasticidin resistance gene containing plasmids and 0,2 µg/ml of puromycin for puromycin resistance gene containing plasmids). At these antibiotic concentrations parental cells are killed and the only cells growing are likely to be those which have integrated in their DNA the transfected plasmid. The plates were then kept at 37°C and the medium renewed every two-three days. When colonies were formed, they were visualized under the microscope and isolated from the plate by using plastic rings which were attached to the plate with Vaseline. Once attached, 20 microliters of a trypsin EDTA solution was introduced into the cloning rings to detach the cells of the single

colony detached and transfer them in 24 wells plates containing 1 ml of medium plus selection antibiotic. The different clones picked up from the original plates are grown and passaged in duplicated in 6 well plates. One plate is used to verify the presence of the gene of interest and the other is used to maintain the clone for further studies and for long term storage. The positive clones could be screened by Western blotting analysis to verify either the downregulation (antisense strategy and siRNA strategy) or the inducible overexpression (dominant negative strategy) of the protein of interest.

The clones overexpressing the inserted gene were then expanded and stored in different aliquots in liquid nitrogen.

2.5 LUCIFERASE REPORTER ASSAY

Transient transfection experiments were performed following the calcium phosphate procedure described in the previous paragraph. These experiments were performed to evaluate the promoter activity of DNA fragments subcloned in appropriate vectors. The constructs used utilize the non mammalian gene luciferase, which is inserted in a promoterless plasmid. For this study pGL2 vector from Promega was used. This plasmid contains the fire fly luciferase gene followed by a viral enhancer and a multiple cloning site at its 5' end. The transfection and transient expression of this plasmid in mammalian cells results in a very low level of luciferase (due to the absence of a promoter), which was detected in cell lysates using a commercially available kit (Dual luciferase system, Promega). The Chk1 genomic fragments to be analyzed were subcloned in the multiple cloning sites of the pGL2 vector using appropriate restriction enzymes. Once the exact insertion and orientation of the fragment was verified using the procedures reported in

paragraph 2.4, the plasmids were transiently transfected into mammalian cells growing in culture. At 48-72 hours following transfection, the medium was removed and the cells processed as reported in the instruction manual of the kit. The luciferase levels were measured in a luminometer and the values corrected for the expression of a control plasmid co-transfected with the plasmid under examination, which encodes a renilla luciferase distinguishable from the fire-fly luciferase utilized in these experiments.

2.6 ANTISENSE STRATEGY AND ANTISENSE CONSTRUCTS

Antisense mRNA is an mRNA transcript that is complementary to an endogenous target mRNA. It basically represents the non coding strand complementary to the coding sequence of mRNA. Introducing a transgene coding for antisense mRNA is a common strategy used to block the expression of a gene of interest although in the last 5 years the more efficient small interfering RNA strategy in large part substituted the antisense strategy.

The availability of the pBluescript (BSSK) plasmid containing the full length human Chk1 cDNA (kindly provided by Yolanda Sanchez) in the MCS, represented the starting point for the construction of three constructs containing fragments of different length cloned in an antisense orientation respect to the sense of transcription in the expression plasmid pcDNA3 (Invitrogen, fig 2.1). Briefly, the 198 bp fragment was obtained by digestion of cDNA Chk1 with the XhoI and BglII restriction endonucleases, subsequently ligated in pcDNA3 previously digested with BamHI (protruding ends compatible with BglII) and XhoI. The 675 bp fragment was obtained by digestion of the cDNA with XhoI and PvuII restriction enzymes and

ligated in pcDNA3 digested with EcoRV (protruding ends compatible with PvuII) and XhoI. The 1,124 bp fragment was obtained by digestion of the cDNA with XhoI and BamHI restriction enzymes and ligated in pcDNA3 digested with Bam HI and XhoI. The subsequent steps of cloning after the ligation were performed as described in paragraph 2.4.

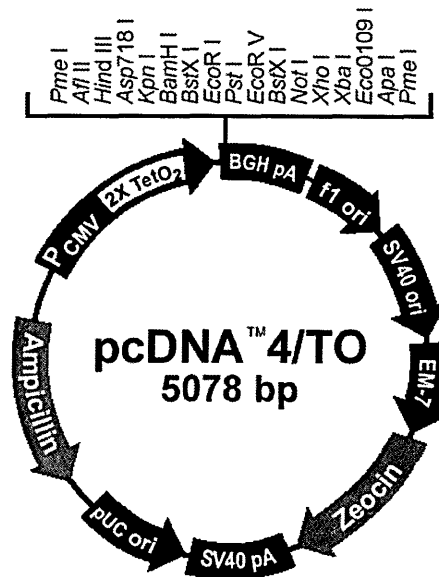
2.7 GENERATION OF THE CONSTRUCT FOR THE INDUCIBLE EXPRESSION OF DOMINANT NEGATIVE CHK1 BY USING THE Trex SYSTEM

To get cellular clones inducibly expressing the Chk1 dead kinase protein tetracycline inducible system (T-Rex System, Invitrogen) was employed. The T-REx System is a tetracycline-regulated mammalian expression system that uses regulatory elements from the E. coli Tn10-encoded tetracycline (Tet) resistance operon (Hillen and Berens, 1994; Hillen et al., 1983). Tetracycline regulation in the T-REx System is based on the binding of tetracycline to the Tet repressor and derepression of the promoter controlling expression of the gene of interest .

The major components of the T-REx System are:

- an inducible expression plasmid (pcDNA4/TO) for expression of the gene of interest under the control of the strong human cytomegalovirus immediate-early (CMV) promoter and two tetracycline operator 2 (TetO2) sites (Figure 2.2A)
- a regulatory plasmid, pcDNA6/TR[®], which encodes the Tet repressor (TetR) under the control of the human CMV promoter (Figure 2.2B)
- tetracycline for inducing expression

A



B

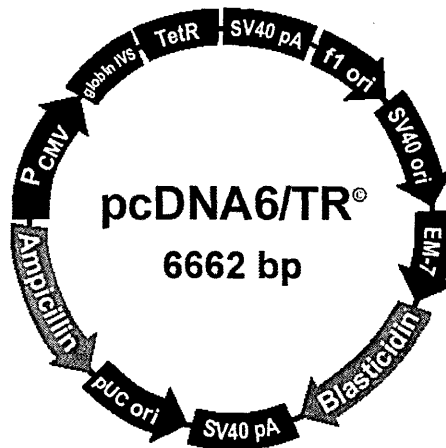


Figure 2.2 Trex inducible expression system: vectors

A. pcDNA4/TO expressing the gene of interest under the control of a hybrid CMV/TetO₂ promoter.

B. pcDNA6/TR expressing the Tet repressor under the control of the human CMV promoter.

In the T-REx System, expression of the gene of interest is repressed in the absence of tetracycline and induced in the presence of tetracycline.

In the absence of tetracycline, the Tet repressor forms a homodimer that binds with extremely high affinity to each TetO2 sequence in the promoter of the inducible expression vector (Hillen and Berens, 1994). The 2 TetO2 sites in the promoter of the inducible expression vector serve as binding sites for 4 molecules (or 2 homodimers) of the Tet repressor. The affinity of the Tet repressor for the tet operator is $K_B = 2 \times 10^{11} \text{ M}^{-1}$ (measured under physiological conditions), where K_B is the binding constant (Hillen and Berens, 1994). Binding of the Tet repressor homodimers to the TetO2 sequences represses transcription of your gene of interest. Upon addition, tetracycline binds with high affinity to each Tet repressor homodimer in a 1:1 stoichiometry and causes a conformational change in the repressor that renders it unable to bind to the Tet operator. The association constant, K_A , of tetracycline for the Tet repressor is $3 \times 10^9 \text{ M}^{-1}$ (Hillen and Berens, 1994). The Tet repressor:tetracycline complex then dissociates from the Tet operator and allows induction of transcription from the gene of interest.

Figure 2.3 shows how all system components work in the cellular context.

2.7.1 CLONING OF THE DOMINANT NEGATIVE CHK1 CONSTRUCT IN PCDNA4/TO

The HCT-116 Trex positive clone transfected with pcDNA6/TR was previously isolated in the laboratory, whereas according to what have been just described, the fusion protein HA-D130 cDNA (Chk1 cDNA with a point mutation at the position 130 in the protein kinase domain which substituted aspartic acid with alanine fused to the

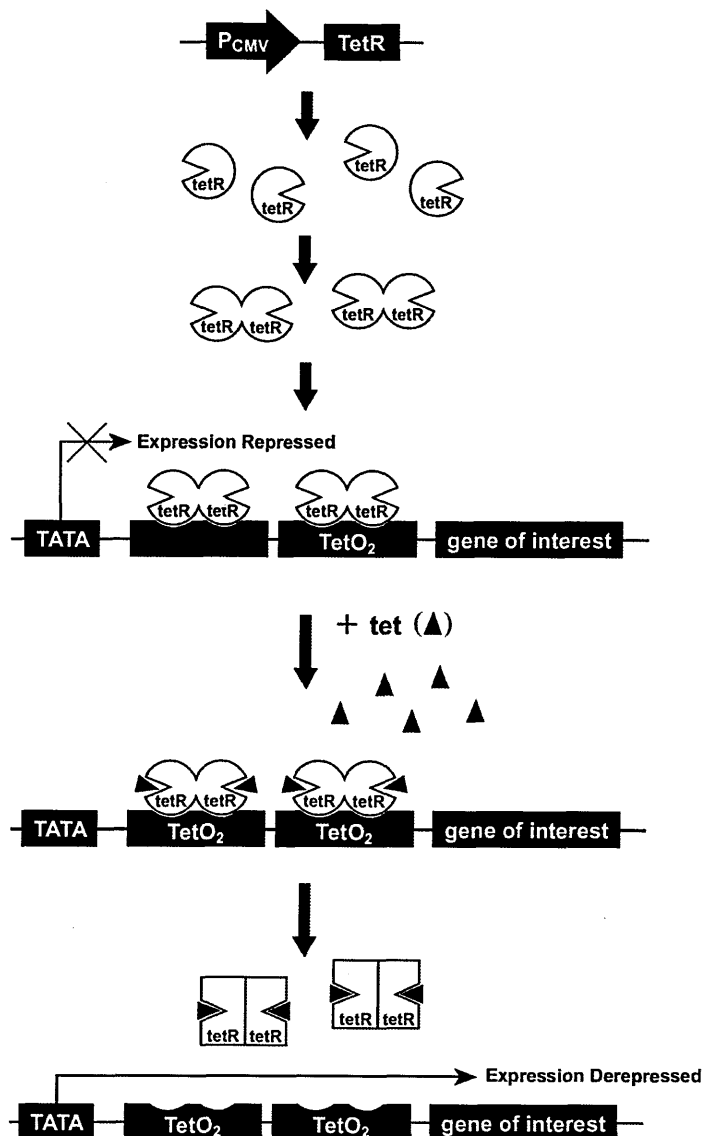


Figure 2.3 T-rex system

The repressor (tetR) protein is expressed from pcDNA6/TR; a homodimer of this protein binds to Tet operator2 ($TetO_2$) in the inducible expression vector. Binding of tet to tetR homodimers causes release from the Tet operator and induction of transcription of the gene of interest.

HA epitope) needed to be subcloned into the eukaryotic expressing vector pcDNA4/TO, in order to be transfected and expressed into eukaryotic cells after Tet induction.

Briefly HA-D130 cDNA prepared by a mini prep from the expression vector pcDNA3-HA-D130 kindly provided by Dr. Ciro Mercurio (IEO, Milan) and the pcDNA4/TO were both digested with HindIII and NotI restriction enzymes for 2 hours at 37 °C. This double digestion allowed the formation of sticky ends between the linearized pcDNA4/TO plasmid and the HA-D130 cDNA fragment. After digestion, the reaction products were loaded onto 1% agarose gel and the expected fragment purified as reported before.

The linearized pcDNA4/TO vector showed a molecular size of 5.4 Kb, HA-D130 cDNA of 1.9Kb and pcDNA3.1 of 5.5 Kb, as expected. pcDNA4/TO vector and HA-D130 were then ligated and the procedures of cloning made as described in paragraph 2.4 of this chapter. A colony positive for the insert of the correct size after digestion with NotI and HindIII was selected and the sequencing was performed (by PRIMM) to check whether the HA-D130 cDNA was ligated in frame for the correct “*in vitro*” synthesis of the HA-D130 protein.

2.8 KO CHK1 CONSTRUCTS

The construction of the two Chk1 KO constructs was possible thanks to the availability of the previously isolated genomic clone 53P13 containing a large part of the Chk1 gene (exons and introns) and by applying the PCR strategy which is herein described.

2.8.1 PCR

The polymerase chain reaction (PCR) follows the principles in which a pair of primers (forward and reverse) defines the region to be amplified. After denaturation (generally at 95 °C), primers are allowed to anneal to their complementary strand by lowering the temperature to their optimal annealing temperature (AT) which is specific for each set of primers and is linked to the oligonucleotides sequence. AT can be calculated on the basis of the following formula: $AT = T_m - t$, where $T_m = 4x (G+C) + 2x (A+T)$ is the melting temperature and $3\text{ }^{\circ}\text{C} > t > 5\text{ }^{\circ}\text{C}$. An elongation step allows the Taq DNA polymerase to start the polymerization reaction downstream of the 3' ends of the primers (generally at a temperature of 72 °C). These three steps are then repeated for “n” (usually 30) cycles to allow exponential amplification of the target sequence. A final step, at 72 °C for 7–10 minutes allows the Taq polymerase to conclude the polymerization step. Amplification is measured by resolving products through agarose gel electrophoresis. Whenever necessary, PCR products were then purified from gel. PCR reaction mix was made using as a Taq purchased from Takara (Gennevilliers, France) with which all the components necessary for the reaction (except for the primers and DNA template) are provided. PCR reaction mix was made following manufacturer's instructions. Generally, nucleotides (dNTPs) are used to a final concentration of 0.2 mM, MgCl_2 to a final concentration of 1.5 mM, and 0.5 U of Taq. Forward and reverse primers were added to a final concentration of 500 nM. The “GeneFisher” software free available online (web address: <http://bibiserv.techfak.uni-bielefeld.de/genefisher/>) was used to design the best set of primers to be used for each region of the gene to be amplified. Synthesis of oligonucleotides used as primers was performed by Sigma.

To the reaction mix, DMSO (dimethyl sulfoxide) was also added to a final concentration of 5%. To the final reaction mixture, a quantity of DNA template was added, (20 ng of plasmidic DNA, 50 ng of genomic DNA) then quickly spun in a microfuge to recover all the fluids at the bottom of the tube and placed in PTC-200 thermal cycler (MJ research, Watertown, MA). Amplification conditions and cycle numbers were selected each time according to the AT of the pair of primers and the length of the PCR product to be amplified.

2.8.2 CLONING OF THE KO CONSTRUCTS

As it is possible to observe from the picture in figure 4.5 of my results, NEO and HYGRO resistance cassettes (previously amplified by PCR) were inserted in between the 5' arm and the 3' arm of each construct with the aim to substitute exon2, intron 2 and exon 3 of the Chk1 gene by homologous recombination. Briefly, to amplify by PCR the 5' arm and the 3' arm, specific primers were designed and specific restriction sites were inserted at the extremities so that the PCR products would have been subsequently cloned in the promoterless pBluescript vector (pBSSK). Before cloning in pBSSK, the PCR amplified fragments were subcloned in the pGEM T easy vector. (see paragraph 2.4.1). Following subsequent step of cloning the entire 5'arm-NEO or HYGRO cassette-3'arm construct was obtained in the pBSSK. The recombinant plasmid was linearized with a unique restriction enzyme (Ahd) and transfected in the HCT-116 cell line, following steps of isolation of positive clones as described in paragraph 2.4.4.

To screen for the positive recombinant clones, genomic DNA was isolated by using the QIAamp DNA Blood Mini Kit (Qiagen) and the manufacturer's instructions were

followed. The screening of positive clones was performed by PCR by using specific primers designed at the extremity of the targeted region, taking advantage to the molecular weight difference between the wt allele and the recombinant allele with the neomycine cassette or the hygromycine cassette present in case of successful event of homologous recombination. The positive clones would show two distinct PCR products with a molecular weight difference of around 400bp.

2.9 RNA INTERFERENCE AND IDENTIFICATION OF A FUNCTIONAL CHK1 SPECIFIC siRNA

RNA interference (RNAi) is the process by which dsRNA (double strand RNA) silences gene expression by inducing the sequence specific degradation of complementary mRNA (siRNA). Long dsRNA is routinely used in non mammalian systems to effect gene silencing. Mammalian cells however have a potent antiviral response pathway that induces global changes in gene expression when dsRNA molecules longer than 30 nt are introduced in the cells (Hammond et al., 2001). A crucial insight came from Tushl and his group in 2001 (Elbashir et al., 2001) by showing that chemically synthesized short dsRNA molecules of 21-22 nucleotides known as small interfering RNA (siRNA) could be used to target mammalian genes by RNAi without activating the interferone response. Briefly the siRNA duplexes are composed of 21-nt sense and 21-nt antisense strands, paired in a manner to have a 2-nt 3' overhang of uridine and a 5'- phosphate and 3' hydroxyl groups. Once transfected in the cells, the siRNAs are incorporated into a multiprotein RNA-inducing silencing complex (RISC) containing a helicase which

unwinds the duplex leaving the antisense strand to guide RISC to its homologous target mRNA for endonucleolytic cleavage as it is shown in figure 2.4.

Subsequent steps were performed to find a working siRNA directed against Chk1.

2.9.1 *IN VITRO SYNTHESIS OF siRNA CHK1*

At first to choose a working siRNA Chk1 I designed different siRNA specific for different Chk1 cDNA targets which were *in vitro* transcribed using the Silencer siRNA construction kit (Ambion). The sequences were chosen following the criteria described in the kit datasheet which had to meet the requirements for the *in vitro* transcription by the T7 RNA polymerase. Briefly the first two nucleotides of the RNA transcript at the 5' ends had to be either GG or GA to ensure efficient synthesis while the two nucleotides at the 3' ends had to be UU. In figure 2.5 the 4 sequences picked up to be *in vitro* transcribed on the Chk1 cDNA are underlined and in particular the working one is outlined in yellow. Before picking up the sequences, the BLAST against EST libraries was performed to ensure that only the gene of interest was targeted. To perform the *in vitro* transcription of the siRNA Chk1 the following steps were carried out.

- siRNA oligonucleotides sense and antisense were designed with a 3' 8-nt sequence complementary to the T7 Promoter Primer provided with the kit in order to ensure the hybridization of the siRNA oligonucleotides to the T7 primer.

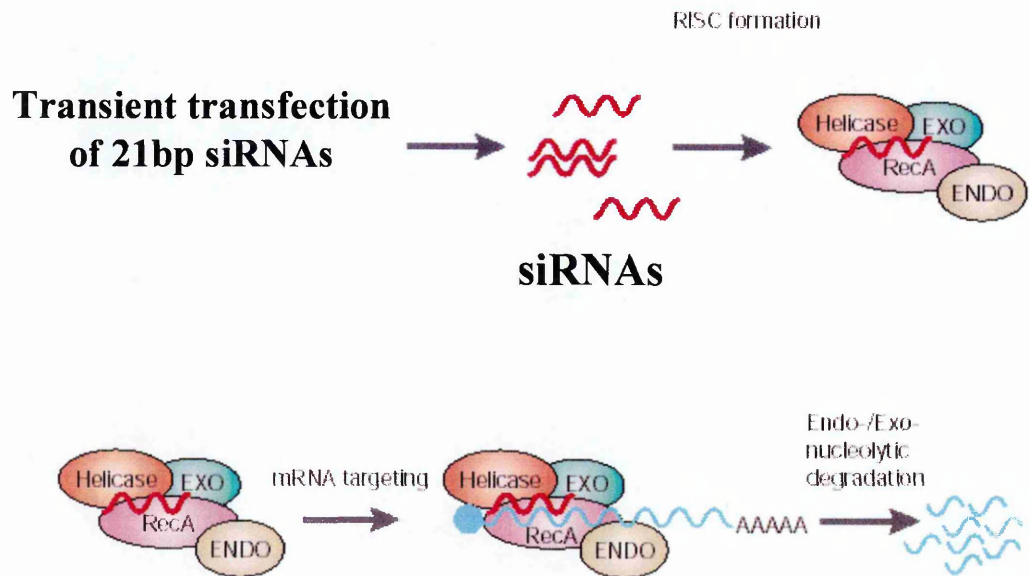


Figure 2.4 mRNA specific degradation by siRNA in mammalian cells

Schematic representation of the mechanism by which siRNAs once transfected in the cells specifically recognize the mRNA target. The RISC complex guides the unwound siRNA to its homologous mRNA target leading to endo and exonucleolytic cleavage and then to mRNA degradation. (Modified by Hammond S.M et al. 2001)

- Oligonucleotides were prepared following the kit instructions and the hybridization reaction with the T7 primer was performed at 70 °C for 5 minutes.
- The 3' ends of the hybridized oligonucleotides were extended by the klenow fragment of DNA polymerase to create double stranded siRNA transcription templates. The reaction occurred at 37 °C for 30 minutes.
- The sense and antisense siRNA templates were transcribed by the T7 RNA polymerase (37 °C for 2 hours) and the resulting RNA transcripts were hybridized to create dsRNA (37 °C overnight).
- The leader sequences were removed by digesting the dsRNA with a single strand specific ribonuclease. Overhanging UU dinucleotides remained on the siRNA because the RNase does not cleave U residues. The DNA template was removed at the same time by a deoxyribonuclease.
- The resulting siRNA had been purified by glass fiber filter binding, following elution which removes excess of nucleotides, short oligomers, proteins and salts in the reaction.
- After quantification by the UV spectrophotometer and before proceeding to transfect the cells, an aliquot of the siRNA product was loaded on a 2% agarose gel in order to test the quality of the 21-nt siRNA isolated.

Figure 2.6 summaries the principal steps of the procedure performed by using the Silencer™ siRNA construction kit (Ambion).

-34 GGCCGGACAGTCCGCCGAGGTGCTCGGTGGAGTCATGGCAGTGCCCTTTGTGGAAGAC
TGGGACTTGGTGCAAACCCTGGGAG **AAGGTGCCTATGGAGAAGTTCA**ACTTGCTGTGA
ATAGAGTAACTGAAGAAGCAGTCGCAGTGAAGATTGTAGATATGAAGCGTGCCGTAGA
CTGTCCAGAAAATATTAAGAAAGAGATCTGTATCAATAAAATGCTAAATCATGAAAAT
GTAGTAAAATTCTATGGTCACAGGAGAGAAGGCAATATCCAATATTTATTTCTGGAGT
ACTGTAGTGGAGGAGAGCTTTTGGACAGAATAGAGCCAGACATAGGCATGCCTGAACC
AGATGCTCAGAGATTCTTCCATCAACTCATGGCAGGGGTGGTTTATCTGCATGGTATTG
GAATAACTCACAGGGATATTAAACCAGAAAATCTTCTGTTGGATGA **AAGGGATAACCT**
CAAAATCTCAGACTTTGGCTTGGCAACAGTATTTTCGGTATAATAATCGTGAGCGTTTGT
TGAACAAGATGTGTGGTACTTTACCATATGTTGCTCCAGAACTTCTGAAGAGAAGAGA
ATTTTCATGCAGAACCAAGTTGATGTTTGGTCCTGTGGAATAGTACTTACTGCAATGGTCG
CTGGAGAATTGCCATGGGACCAACCCAGTGACAGCTGTCAGGAGTATTCTGACTGGAA
AGAAAAAAAACATACCTCAACCCTTGGAAAAAAATCGATTCTGCTCCTCTAGCTCTG
CTGCATAAAATCTTAGTTGAGAATCCATCAGCAAGAATTACCATTCCAGACATCAAAA
AAGATAGATGGTACAACAAACCCCTC **AAGAAAGGGGCAAAAAGGCCCC**GAGTCACTT
CAGGTGGTGTGTCAGAGTCTCCCAAGTGGATTTTCTAAGCACATTCAATCCAATTTGGAC
TTCTCTCCAGTAAACAGTGCTTCTAGTGAAGAAAATGTGAAGTACTCCAGTTCTCAGCC
AGAACCCCGCACAGGTCTTTTCTTATGGGATACCAGCCCTCATACATTGATAAATTGG
TACAAGGGATCAGCTTTTCCCAGCCCATATGTCCTGATCATATGCTTTTGAATAGTCAG
TTACTTGGCACCCCAAGATCCTCACAGAACCCCTGGCAGCGGTTGGTCAAAAGAATGA
CACGATTCTTTACCAAATTGGATGCAGACAAATCTTATCAATGCCTGAAAGAGACTTGT
GAGAAGTTGGGCTATCAATGGAAGAAAAGTTGTATGAATCAGGTACTATATCAACAA
CTGATAGGAGAAACAATAAACTCATTTTCAAAGTGAATTTGTTAGAAATGGATGATAA
AATATTGGTTGACTTCCGGCTTTCT **AAGGGTGATGGATTGGAGTTCA**AGAGACACTTCC
TGAAGATTAAAGGGAAGCTGATTGATATTGTGAGCAGCCAGAAGGTTTGGCTTCCTGC
CACATGATCGGACCATCGGCTCTGGGGAATCCTGGTGAATATAGTGCTGCTATGTTGAC
ATTATTCTTCCTAGAGAAAGATTATCCTGTCTGCAAACTGCAAAATAGTAGTTCCTGAAG
TGTTCACTTCCCTGTTTATCCAAACATCTTCCAATTTATTTTGTGTTGTTCCGCATACAAA
TAATACCTATATCTTAATTGTAAGCAAACTTTGGGGAAAGGATGAATAGAATTCATTT
GATTATTTCTTCATGTGTGTTTAGTATCTGAATTTGAAACTCATCTGGTGGAACCAAG
TTTCAGGGGACATGAGTTTTCCAGCTTTTATACACACGTATCTCATTTTTATCAAAACAT
TTTGTTT +1,787

Figure 2.5 Chk1 cDNA targets for the siRNAs *in vitro* transcription

In figure the four cDNA target sequences chosen for the siRNA *in vitro* transcription are represented in blue. Yellow box shows the cDNA target of the siRNA that most efficiently inhibits the Chk1 expression.

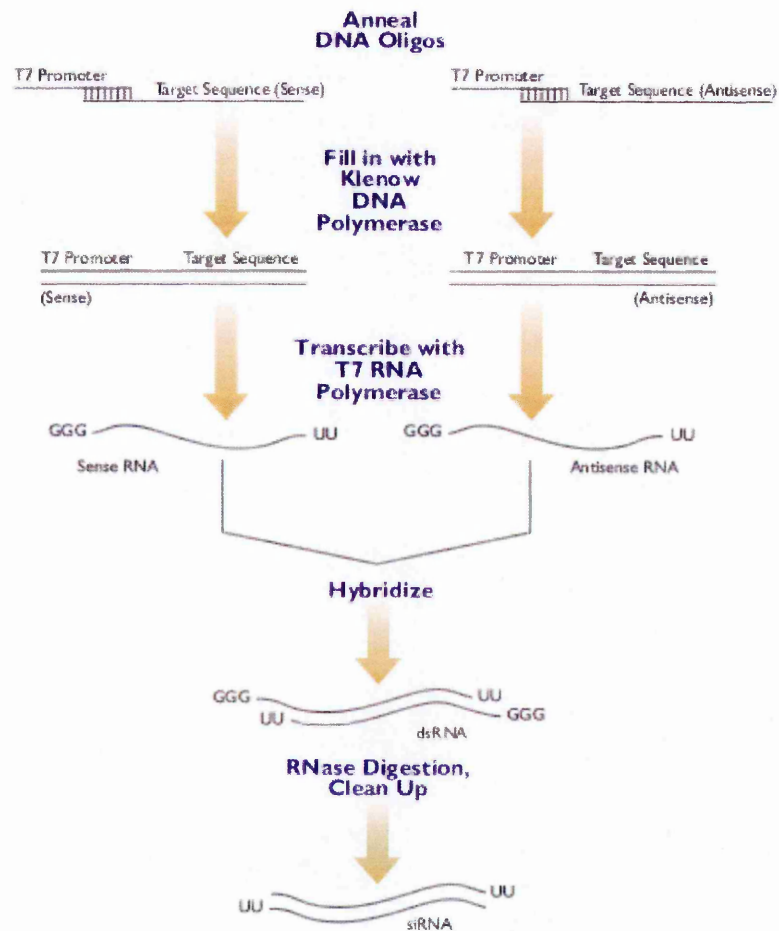


Figure 2.6 siRNA *in vitro* transcription procedure

Schematic representation of the principal steps performed to *in vitro* transcribe the siRNAs by using a T7 RNA polymerase. More details in the text.

2.9.2 SYNTHETIC siRNAs AND TRANSFECTION PROCEDURES

The cDNA Chk1 target sequence comprising nucleotides 420-440 from starting ATG of Chk1 cDNA was found to efficiently inhibit the Chk1 expression and was chosen to be chemically synthesized in a larger scale and used to perform siRNA transfection experiments in the cells. The best siRNA transfection reagent was found to be Lipofectamine 2000 (Invitrogen). At first different concentrations of siRNA were tested. The 60 nM concentration was then found to be the lowest concentration used which ensured a good downregulation of the protein and was subsequently used to perform all the siRNA transfection experiments which were set up in the 24 well plates. Briefly, 60nM of siRNA was mixed with 50 μ l of OPTI-MEM each sample. The solution was subsequently mixed with 1 μ l + 50 μ l of OPTI-MEM, previously incubated at RT for 5 minutes. (NOTE: the amount of lipofectamine in each sample changed depending on the cellular line to be transfected ranging from 1-to 1,3 μ l). The mixture was then incubated for 20 minutes at room temperature and 100 μ l of transfection reaction was added to 500 μ l of media present in the well. The incubation with the transfected solution continued till the end of the experiment.

The synthetic siRNA Chk1 was purchased from Proligo-SIGMA while the validated siRNA Chk2 was purchased from Xeragon oligoribonucleotides (Qiagen). The siRNA Chk1 transient transfection experiments described in chapter 5 were performed by using a modified siRNA sequence directed against the same target sequence previously identified. I introduced an asymmetry in 5' of the antisense strand by introducing a mismatch (nucleotide substitution in 3' of the sense sequence). According to what Zamore and his group have reported in a recent paper, small changes in siRNA sequence

| | | |
|--|--------|---|
| 5' GGGUA AACCUC AAAAUCUCUU 3' |> | siRNA Chk1 personally validated (1) |
| 3' UUCCCUAUUGGAGUUUUAGAG 5' | | |
| 5' GGGUA AACCUC AAAAUCU A UU 3' |> | siRNA Chk1 personally validated (1) with mismatch |
| 3' UUCCCUAUUGGAGUUUUAG G 5' | | |
| 5' GCGUGCCGUAGACUGUCCA UU 3' |> | siRNA Chk1 validated by Zhao et al. (2) |
| 3' UUCGCACGGCAUCUGACAGGU 5' | | |
| 5' ACUAUAAGGGUCAACACCUUU 3' |> | scramble siRNA (1) |
| 3' UUUGAUAUUCCAGUUGUGGA 5' | | |
| 5' GCUUCCCAGUGGCAGGUCAUU 3' |> | scramble siRNA (2) |
| 3' UUCGAAGGGUCACCGUCCAGU 5' | | |
| 5' CGCCGUCCUUUGAAUAACA UU 3' |> | siRNA Chk2 validated by Xeragon |
| 3' UUGCGGCAGGAAACUUAUUGU 5' | | |

Figure 2.7 siRNA duplex sequences

A list of the sequences of siRNAs used in this thesis is provided in the figure. In the second duplex the mismatch introduced is marked with red colour.

should have big effects on the participation of each individual strand of a siRNA duplex in entering the RNAi pathway. The RISC assembly is guided by an enzyme that selects which strand of siRNA is loaded into the RISC whereas the other one is degraded. This strand is usually the one whose 5' end is less paired to its complementary. Thus the mismatch in 5' of the antisense was designed to meet the biological requirements for the entry of the antisense strand into the RISC in order to render more efficient the downregulation (Schwarz et al., 2003). The second Chk1 cDNA target sequence used in the siRNA double transfection system was the validated one by Piwnicka-Worms and her group (Zhao et al., 2002) and the cDNA target sequence comprises nucleotides 127-147 from starting ATG. The control scramble siRNAs directed against the two siRNA Chk1 sequences were designed by using a proligo database.

In figure 2.7 the siRNA duplexes sequences used in this thesis are listed.

2.9.3 *siRNA CHK1 INDUCIBLE EXPRESSION*

To get cellular clones inducibly expressing the siRNAs against Chk1 the pSuperior plasmid (Oligoengine) was used (figure 2.8A). This plasmid basically has the same role that the pcDNA4/TO has in the inducible over-expression of a protein of interest. Briefly, the siRNA target was subcloned in the pSuperior plasmid downstream to an H1 promoter which is responsive to the Tet repressor expressed by the second plasmid, pcDNA6/TR. In the absence of tetracycline in the culture media, the Tet repressor binds to the promoter avoiding the expression of the siRNA. In the presence of tetracycline the Tet repressor detaches and the H1 promoter gets activated expressing the siRNA of interest at first in a structure of a hairpin which permits the annealing of the sense and

antisense sequences and then leading to the formation of the double strand RNA once the loop is cut by intracellular specific enzymes (figure 2.8B).

To clone the single cDNA target for the siRNA of interest in the pSuperior plasmid the following steps were performed:

- 59-nt forward and reverse oligonucleotides were designed with the following structure each:
 - BglIII (forward) and HindIII (reverse) restriction site in 5'
 - Target sense of 19-nt
 - Hairpin of 9-nt
 - Target antisense of 19-nt
 - Termination signal consisting of five thymidines in a row (T5) (the cleavage of the transcript at the termination site is after the second uridine so that the two 3' overhanging U nucleotides are present as in the synthetic siRNAs. This region is inserted in 3' of the forward oligonucleotide and in 5' immediately after the HindIII site of the reverse oligonucleotide.
- The forward and reverse siRNA oligonucleotides were annealed in Universal Buffer (100 mM NaCl, 50 mM Hepes pH 7.4) by incubating 4 minutes at 94 °C and then at 70 °C for 10 minutes following a progressive cooling step (37 °C for 15-20 minutes and at room temperature before using). In the mean time the pSuperior vector was cut with BglIII and HindIII
- The annealed oligonucleotides were cloned into the linearized vector and the subsequent cloning steps were performed as described in paragraph 2.4.

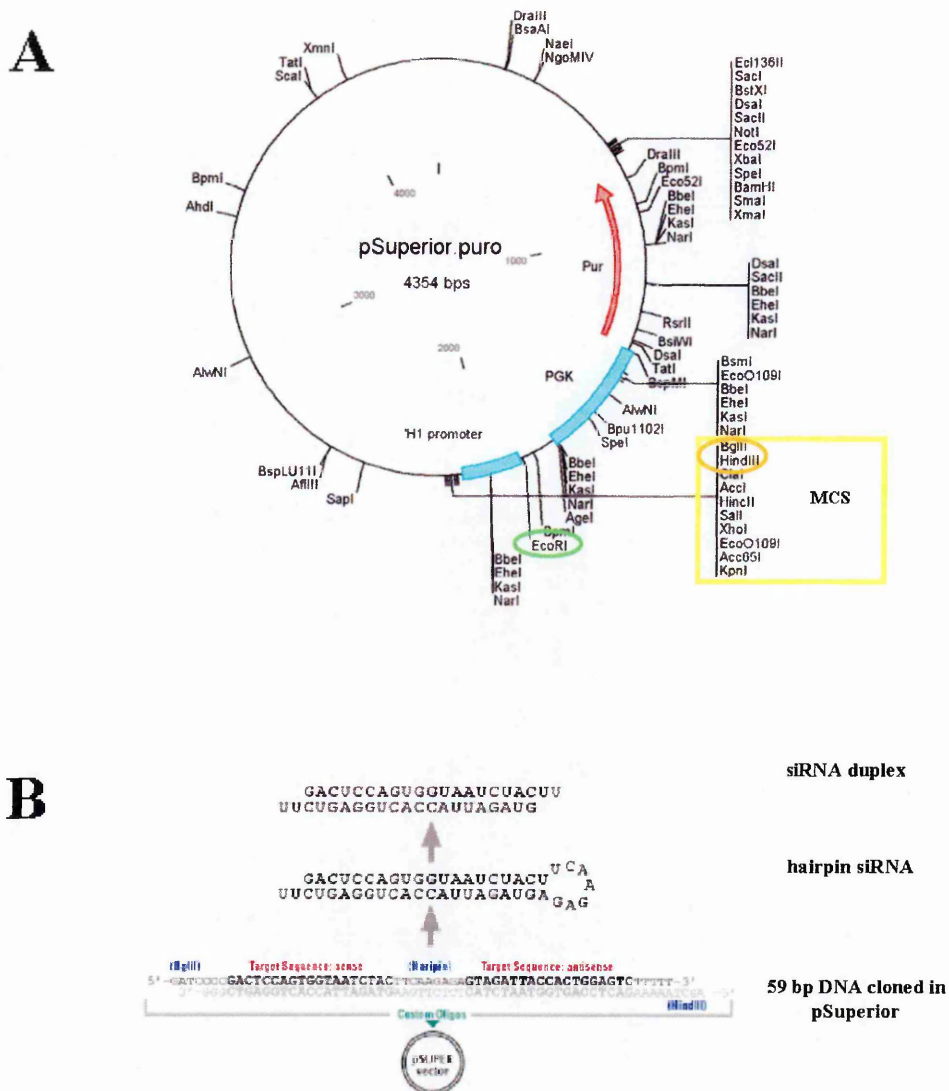


Figure 2.8 siRNA inducible expression system

- A. pSuperior/puro vector. The two restriction enzymes chosen for cloning, BglII and HindIII are underlined in the MCS. EcoRI restriction site (used for the double siRNA cloning) in 5' of the H1 promoter is also underlined.
- B. Schematic representation of the siRNA duplex formation in the cells from the DNA of 59bp cloned in the pSuperior vector.

To clone the second cDNA target for the siRNA of Chk1 in the pSuperior already containing the first cDNA Chk1 target, the forward and reverse oligonucleotides of this second target had to be designed by adding downstream to the termination sequence, the EcoRI restriction site, so that after the first step of cloning by following the same cloning procedures just described, the target sequence inserted together with the H1 promoter could be excised by cutting with the EcoRI restriction enzyme and then ligated in the first pSuperior containing one cDNA Chk1 target and previously linearized with EcoRI (single site in the vector).

To clone the siRNA scramble sequences the same procedures just described were followed.

2.10 IDENTIFICATION OF TRANSCRIPTIONAL STARTING SITES

To identify the Chk1 gene transcriptional starting sites the primer extension assay was performed. The first necessary step was the isolation of mRNA from the total RNA. The total RNA was isolated from the HCT-116 cell line as previous data in literature (Damia et al., 2001) had attested to have the two different length transcripts of Chk1.

2.10.1 TOTAL RNA EXTRACTION

The SV total RNA isolation kit (Promega) was used for isolating total RNA. By using this method it is possible to isolate pure RNA from relatively few cells (10^6) in a limited time (1-2 hours). The cells are lysed in a guanidine thiocyanate containing solution which maintains the integrity of RNA while disrupting cells and dissolving cell

components. RNA was then prepared following exactly the manufacture's instructions. The purified RNA is then eluted with a small volume of water (generally 50 microliters). An aliquot (2 μ l) of this solution was diluted in sterile water to a final volume of 200 μ l, and the RNA concentration was measured spectrophotometrically following the formula $\text{RNA (mg/ml)} = \text{OD}_{260 \text{ nm}} \times 40 \times \text{dilution factor}$. The amount of RNA is calculated considering that a solution of 40 μ g of RNA in one ml would give an absorbance reading of 1.0 at 260 nm. The 260/280 absorbance ratio must be between 1.8 and 2.0 for a sample of reasonable purity. Samples were stored at -80°C until used.

2.10.2 PURIFICATION OF POLY A⁺ RNA FROM TOTAL RNA PREPARATIONS

To isolate mRNA from the total RNA previously extracted, the Dynabeads Oligo (dT)₂₅ Kit from Dynal (Invitrogen) was used and the procedure followed is herein described. Briefly, the use of Dynabeads Oligo (dT)₂₅ relies on base pairing between the poly(A⁺) residues at the 3' end of most messenger and the oligo d(T) residues covalently coupled to the surface of the Dynabeads Oligo (dT)₂₅.

Around 75 μ g of total RNA was adjusted to a total volume of 100 μ l with distilled DEPC treated water. The same total volume of 2X Binding Buffer (solution B) was then added to the Dynabeads Oligo(dT)₂₅ to obtain a 1X binding buffer concentration in order to get the most optimal binding concentration.

2X Binding Buffer (solution B):

20mM Tris-HCl (pH 7.5)

1M LiCl

2mM EDTA

200µl of resuspended Dynabeads Oligo(dT)₂₅ were then transferred from the stock tube suspension to an RNase free Eppendorf tube placed in a Dynal MPC which is an apposite magnetic particle concentrator rack. After 30 seconds or when the suspension was clear the supernatant was removed and the dynabeads were washed by resuspension with 100µl of 2X binding buffer. The 2X binding buffer was then removed from the dynabeads while the vial is placed in the dynal MPC then the vials were removed from the Dynal MPC. The dynabeads were resuspended again with 100µl of 2X Binding buffer. The total RNA suspension which was previously heated at 65 °C for 2 minutes to disrupt secondaries structures, was then added to the Dynabeads suspension. The mixture was mixed gently and the annealing was assured by rotating on a roller for 3-5 minutes at room temperature. The vials were then placed in the Dynal MPC for 30 seconds and the supernatant then removed. The vials were transferred to another rack and washed with 200 µl of Washing buffer (solution E) using the Dynal MPC. The mRNA was then eluted from the Dynabeads by adding 10-20 µl of Elution Buffer (solution F) and by keeping them at 65 °C for 2 minutes. The tubes were then immediately placed in the Dynal MPC and the supernatant containing the mRNA was transferred in a new RNase free tube. It was possible to calculate the concentration of the isolated mRNA by aliquoting 2 µl of the total solution in sterile water to a final volume of 200 µl, and the mRNA concentration was measured spectrophotometrically following the formula $\text{RNA (mg/ml)} = \text{OD}_{260 \text{ nm}} \times 40 \times \text{dilution factor}$. The amount of mRNA is calculated considering that a solution of 40 µg of RNA in one ml would give an absorbance reading of 1.0 at 260 nm. The 260/280 absorbance ratio must be between 1.8 and 2.0 for a sample of reasonable purity. Samples were stored at -80°C until used.

Washing buffer (solution E)

10mM Tris HCl (pH 8.0)

0.15M LiCl

1mM EDTA

Elution Solution (solution F)

10mM Tris pH 7.5

2.10.3 PRIMER EXTENSION REACTION

Briefly, the primer extension analysis is used to determine the location and to quantitate the amount of 5'-end of specific RNAs. An end labeled oligonucleotide is hybridized to RNA and is utilized as a primer by reverse transcriptase in the presence of deoxynucleotides. The RNA is reverse transcribed into cDNA and is analyzed on a denaturing polyacrylamide gel. The length of the cDNA reflects the number of bases between the labeled nucleotide of the primer and the 5' -end of the RNA whose length in our experiment was calculated thanks to the DNA sequence loaded close to the primer extension reaction in the same denaturing polyacrylamide gel. In our specific investigation the transcriptional starting site upstream to the second exon was found thanks to the utilization of a specific primer located in the second exon, called CHP2 (figure 2.9).

2.10.3.1 OLIGONUCLEOTIDE LABELING BY PHOSPHORYLATION

The following components were mixed to a 1,5 ml microcentrifuge tube:

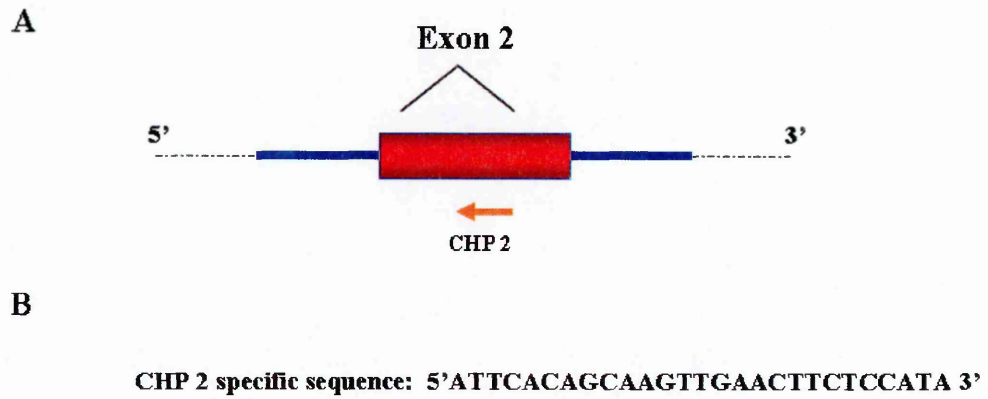


Figure 2.9 CHP2 primer used for primer extension reaction

- A. Schematic representation of the primer's position in the Chk1 gene.
- B. Specific CHP2 primer sequence.

| | |
|--|---------------------|
| <i>Oligonucleotide primer (1μM)</i> | <i>5μl (5pmol)</i> |
| <i>10X T4 Polynucleotide Kinase Buffer</i> | <i>2 μl</i> |
| <i>[γ- ³²P] ATP(10mCi/ml)</i> | <i>3 μl (30μCi)</i> |
| <i>T4 Polynucleotide Kinase</i> | <i>1 μl</i> |
| <i>H₂O</i> | <i>9 μl</i> |
| <i>Total</i> | <i>20 μl</i> |

The mixture was incubated at 37 °C for 45-60 minutes, following the inactivation of the kinase for 10 minutes at 68 °C .

Purification of the oligonucleotide from unlabelled nucleotide was performed using Sephadex G50 columns (Boheringher-Mannheim) exactly following the manufacturer's instructions. Briefly the column was at first equilibrated by centrifuging for 2 minutes at 3,000 rpm; then the 20 μl of reaction were entirely loaded in the column and centrifuged for 4 minutes at 3,000 rpm. The incorporated radioactivity was detected by Cherenkov counting. The saved oligonucleotide could be stored at – 20 °C, being stable for up to 1 month.

2.10.3.2 HYBRIDIZATION OLIGO/RNA

The following components were mixed to a 1,5 ml microcentrifuge tube:

| | |
|-------------------------------|----------------------------|
| <i>mRNA 1μg/μl</i> | <i>3 μl</i> |
| <i>Buffer 10X</i> | <i>1,5 μl</i> |
| <i>Probed oligonucleotide</i> | <i>at least 50,000 cpm</i> |
| <i>H₂O</i> | <i>to 15 μl</i> |

The mixture was incubated at 60 °C for 90 minutes then it was cool down at room temperature for at least 15 minutes.

2.10.3.3 PRIMER EXTENSION REACTION

To each previous oligo/RNA annealed mixture the following components were added:

| | |
|------------------------------|-----------------|
| <i>Tris HCl pH 8.3 1M</i> | <i>0,9µl</i> |
| <i>MgCl₂ 0,5M</i> | <i>0,9µl</i> |
| <i>DTT 1M</i> | <i>0,25µl</i> |
| <i>Actinomycin D</i> | <i>6,75 µl</i> |
| <i>dNTP 2,5mM</i> | <i>2,66 µl</i> |
| <i>superscript (25U/ µl)</i> | <i>0,2 µl</i> |
| <i>H₂O</i> | <i>to 30 µl</i> |

The mixture was incubated at 42 °C for 1 hour then 1/10 volume of sodium acetate 3M and 2-3 volumes of ethanole 100% was added for precipitation by incubating 15-30 minutes in dry ice and ethanole 100%, following a 30 minutes centrifugation at 13,000 rpm. The samples were desiccated by speed vacuum and then resuspended in 5 µl of loading dye buffer, following boiling for 2 minutes to denature DNA-RNA hybrids and then loading on the denaturing polyacrylamide gel together with a DNA sequenced sample to detect the product's length.

2.10.3.4 DENATURING GEL PREPARATION

Denaturing gel recipe:

final concentration

| | |
|--|------------|
| <i>15 ml 30% acrylamide/bis 37.5:1</i> | <i>6%</i> |
| <i>18 ml TBE 5x</i> | <i>1x</i> |
| <i>37.8 g urea</i> | <i>7 M</i> |

Basically, urea was dissolved in the acrylamide solution with TBE 1x and the final volume made up to 90 ml with distilled water. The solution was placed on ice to cool before the addition of 10% of ammonium persulphate (450 µl) and TEMED (15 µl) just before pouring to give a gel of dimensions 30x40 cm. Sharks tooth (14 cm) combs were put in upside down and the gel was left to polymerise for about 2 h. The gel was placed in a model S2 sequencing gel electrophoresis apparatus (Gibco-BRL-Life Technologies, Milan-Italy). 1x TBE buffer was used as the running buffer. The gel was pre-electrophoresed for 30 min at 60 W, using a LKB power pack (Hoefer, Life Technologies).

TBE (5x) recipe:

| | <u><i>final concentration</i></u> |
|-------------------|-----------------------------------|
| 54 g Tris base | 446 mM |
| 27.5 g boric acid | 445 mM |
| 4.65 g EDTA | 12 mM |

to a final volume of 1 l with distilled water. Solution was stored at room temperature.

2.10.3.5 DNA SEQUENCING

To find out the length of the band obtained by the primer extension reaction a DNA sequence ladder was loaded in parallel on the acrylamide denaturing gel and was prepared following the Sanger sequencing protocol. The Sanger sequencing protocol is generally known as the chain termination method (Sanger et al., 1992). Chain termination is achieved by incorporating a nucleotide analogue at the end of the growing DNA strand that lacks a 3'-hydroxyl group for attachment of the next nucleotide. This is done by individually introducing a small amount of the 2', 3'-dideoxyribonucleotide-5'-triphosphate (ddNTP) for one of the four DNA bases into each of the four DNA synthesis reaction mixtures described above. The dideoxy analogs are incorporated onto the 3'-ends of the growing chains like normal deoxynucleotides, but they lack the 3'-hydroxyl group needed for addition of the next nucleotide. Thus, whenever one of them is randomly incorporated, growth of the chain is irreversibly terminated at that point. In the Sanger protocol, a purified DNA polymerase with no primase activity is used, thus preventing any possible spontaneous initiation of synthesis at unwanted locations. Priming is done with a synthetic oligonucleotide that is complementary to a specific sequence on the template strand. Plasmid DNA was denatured with NaOH (2 M) for 30 min at 37°C. DNA was precipitated by addition of 2.5 volumes of 100% ice-cold ethanol and 10% 3 M NaAc, and left on dry ice for 30 min and then centrifuged for 30 min at 15,000xg. The pellet was washed with 200 µl of 75% ethanol and centrifuged as before for additional 4 min. The pellet was air dried and resuspended in 8 µl of sterile water. T7 (5 pmol) primer was added and the final preparation of the sample was accomplished using α -(³⁵S) dATP and "Sequenase Version 2" sequencing kit (Amersham-Life Science). A radioactive labelled nucleotide was included in the synthesis so that labelled

chains of various lengths can be visualised by autoradiography after separation by high-resolution electrophoresis.

2.11 PROTEIN ANALYSIS: WESTERN BLOTTING

Western blot analysis ensures separation of proteins according to size using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE), transferring them to nitro-cellulose electrophoretically and then using specific antibodies to detect the protein of interest.

2.11.1 PROTEIN EXTRACTS PREPARATION

Total proteins were extracted from cells growing in culture by a lysis method. Basically cell cultures were washed twice with ice-cold PBS and then detached with a disposable scraper in 500 µl of PBS. The suspension was then centrifuged at 1,200 rpm for 10 minutes and after centrifugation, the pellet was resuspended in an amount of lysis buffer dependent on the size of cell pellet

| <i>Lysis Buffer</i> | <i>final concentration</i> |
|-------------------------------|----------------------------|
| <i>20 µl Triton X-100</i> | <i>1%</i> |
| <i>20 µl Tris 1M, pH 7.4</i> | <i>10 mM</i> |
| <i>60 µl NaCl 5 M</i> | <i>150 mM</i> |
| <i>8 µl leupeptin 5 mg/ml</i> | <i>20 µg/ml</i> |
| <i>5 µl aprotinin 2 mg/ml</i> | <i>5 µg/ml</i> |
| <i>20 µl PMSF 100 mM</i> | <i>1 mM</i> |

to a final volume of 2 ml with sterile water.

and incubated on ice for 30 minutes in an orbital shaker. After 10 min of centrifugation at 12,000 x g at 4°C, cellular debris were pelleted and the total protein present in the supernatant was recovered and placed in a fresh Eppendorf tube (1.5 ml). An aliquot (2 µl) was used for determination of protein concentration.

2.11.2 CALIBRATION CURVE PREPARATION

Solutions of bovine serum albumin (BSA) (ranging between 1 and 20 µg/200 µl) were prepared from a stock solution of BSA obtained by dissolving powdered BSA (Sigma) in water. In a 1.5 ml tube, 200 µl of each BSA solution were mixed with 600 µl of distilled water and 200 µl of BioRad protein assay dye (BioRad). In the blank sample 800 µl of distilled water was mixed with 200 µl of BioRad protein assay dye. Samples were rapidly transferred into disposable cuvettes (PBI International, Milan-Italy) and the absorbance at 595 nm was measured in the spectrophotometer. The absorbance value corresponding to the blank sample was subtracted from the values obtained in the BSA-containing samples. Each calibration sample was run in triplicate. The calibration curve obtained in such a way, allows extrapolation of the exact absorbance value corresponding to 1 µg of proteins present in the solution.

2.11.3 DETERMINATION OF PROTEIN CONCENTRATION IN CELLULAR EXTRACTS

Protein concentration in the cellular extract was determined according to the Bradford protocol. The concentration of proteins in the samples was determined by mixing in a

1.5 ml tube 2 μ l of protein extract with 200 μ l of BioRad protein assay dye and distilled water in a final volume of 1 ml. Samples were processed as for the calibration curve and the amount of proteins calculated from the absorbance value corresponding to 1 μ g of proteins (obtained from the calibration curve).

2.11.4 SDS-PAGE

An aliquot (typically 30 μ g) of protein of each sample was mixed with the same amount of 2X SDS loading buffer:

| <i>SDS Loading Buffer (2X)</i> | <i>final concentration</i> |
|---|----------------------------|
| 200 μ l Tris 1 M, pH 8.8 | 100 mM |
| 400 μ l DTT 1 M | 200 mM |
| 800 μ l SDS 10% | 4% |
| 400 μ l glycerol | 20% |
| 40 μ l bromophenol blue 10% | 0.2% |
| <i>to a final volume of 2 ml with sterile water</i> | |

and the mixture was boiled for 5 min. Samples were loaded onto a 5% stacking gel and a range of 6-15% separating gel:

| <i>Stacking Gel</i> | <i>final concentration</i> |
|-------------------------------|----------------------------|
| 2.5 ml Tris-HCl 0.5 M, pH 6.8 | 125 mM |
| 0.1 ml SDS 10% (w/v) | 0.1% (w/v) |

1.62 ml 30% acrylamide/bis 37.5:1 *5% (w/v)*

To a final volume of 10 ml with deionised water.

50 µl ammonium persulphate 10% (w/v) *0.05% (w/v)*

5 µl TEMED

Separating Gel *final concentration*

5.0 ml Tris-HCl 1.5 M, pH 8.8 *750 mM*

0.2 ml SDS 10% (w/v) *0.1% (w/v)*

X ml 30% acrylamide/bis 37.5: 1 *X% (w/v)*

To a final volume of 10 ml with deionised water.

100 µl ammonium persulphate 10% (w/v) *0.05% (w/v)*

7.5 µl TEMED

Stacking and separating gels were prepared shortly before pouring. Ammonium persulphate catalyses polymerization and TEMED accelerates the reaction and so these two reagents were added last.

Proteins were resolved on a minigel apparatus (BioRad) and run for 2 h at 100V in 1X TGE buffer:

Running Buffer (TGE 1X) *final concentration*

15.15 g Tris base *25 mM*

72 g glycine *192 mM*

5 g SDS *0.1% (w/v)*

to a final volume of 5 l with deionised water. The buffer was stored at room temperature.

Electrophoresis progress was followed using pre-stained molecular weight markers (11-170 Kda, Page Ruler Prestained Protein Ladder, FERMENTAS)

2.11.5 PROTEIN TRANSFER AND DETECTION

The separated proteins were transferred onto nitro-cellulose (at 60V for 2 h) using BioRad Mini transfer blot equipment in 1X transfer buffer:

| <i>Transfer Buffer 1X</i> | <i>final concentration</i> |
|---------------------------|----------------------------|
| <i>24.2 g Tris Base</i> | <i>50 mM</i> |
| <i>28.5 g glycine</i> | <i>100 mM</i> |
| <i>4 ml SDS 10%</i> | <i>0.01%</i> |
| <i>800 ml methanol</i> | <i>20%</i> |

to a final volume of 4 l with deionised water. The solution was stored at room temperature.

Filters were stained with Ponceau red solution (Sigma) to check sample loading and transfer. Blots were placed in a bag with 5% non-fat dried milk dissolved TBS-T 0.1%

| <i>TBS-T 0.1%</i> | <i>final concentration</i> |
|-------------------------|----------------------------|
| <i>2.42 g Tris base</i> | <i>20 mM</i> |
| <i>8 g NaCl</i> | <i>137 mM</i> |
| <i>1 g Tween 20%</i> | <i>0.1% (w/v)</i> |

to a final volume of 1 l with deionised water. The pH was adjusted to 7.6 with concentrated HCl and stored at 4°C.

and shaken o/n at 4°C to block non-specific binding. All the following procedures were carried out at room temperature on a shaker. Blots were exposed for 2 h at room temperature to the desired primary antibodies diluted to the optimal working solution in TBS-T 0.1%. After incubation, the blots were washed twice with TBS-T 0.1% and incubated with the appropriate horseradish-peroxidase linked anti-mouse or anti-rabbit IgG secondary antibody (Amersham) for 1 h using appropriate dilutions. Blots were washed as previously described, and detection was performed with an enhanced chemiluminescent detection system (ECL, Amersham-Life Science). Briefly, the horseradish peroxidase acts as a catalyst for the oxidation of a luminol substrate, which subsequently emits small but sustained quantities of light. This chemiluminescence is specifically enhanced allowing an image to be recorded on photosensitive film. The blots were exposed to film for different time ranging from 15" to 3 min and developed using an X-o graph compact x-2 developer with Kodak GBX developer and fixer.

The antibodies used for Western blot analysis in this study were:

- Chk1, Chk2, E2F1, cyclin B1, caspase-3, actin and β -tubulin antibodies.

Mouse monoclonal IgG for Chk1, cyclin B1 and caspase-3, rabbit polyclonal IgG for E2F1, Chk2 and β -tubulin and goat polyclonal IgG for actin detection were supplied by Santa Cruz Biotechnology. Stock solutions were stored at 4°C and working solutions were prepared by diluting the antibody 1:500 in 5% non-fat dried milk dissolved in TBS-T 0.1%. Goat anti-mouse for Chk1, cyclin B1 and caspase-3, goat anti-rabbit for

E2F1, Chk2 and β -tubulin and donkey anti-goat for actin IgGs horseradish peroxidase secondary antibodies were supplied by Santa Cruz and diluted 1:3,000 in 5% non-fat dried milk dissolved in TBS-T 0.1%.

2.12 KINASE ASSAY

Chk1 kinase activity was determined after immunoprecipitation with anti Chk1 antibody (G4, Santa Cruz biotechnology). Briefly 100 μ g of protein extracts (prepared as just described) were incubated at 4°C with 1 μ g of anti Chk1 antibody (SantaCruz Biotechnology) overnight with continuous, gentle rocking. To this solution, 50 μ l of agarose beads (protein A/G Santa Cruz Biotechnology) were added and the incubation continued for 1 hour. Agarose bound kinase was collected by centrifugation at 2,000 rpm in an eppendorf tube for 5 min and washed twice with 500 μ l of PBS.

Kinase reaction was performed in 25 μ l reaction containing: 1.5 μ g of recombinant GST-Cdc25C (aa 250-256) previously exratced from bacteria as I will describe in the next paragraph 5 μ Ci of 32 P- γ ATP (Amersham), 1 μ M ATP, 0.5 mM DTT, 50mM Tris HCl, 10 mM MgCl₂. After 20 minutes incubation at 30°C, 25 μ l of 2x loading buffer are added and the mixture heated at 90°C for 5 minutes, cooled on ice and loaded on 10% SDS-PAGE gels.

The gel was run for 16 hours at 30 V after which the gel is removed and directly exposed to autoradiographic films to detect phosphorylated Cdc25C.

2.12.1 PURIFICATION OF GST-FUSION PROTEIN GST-CDC25C FROM BACTERIA

To recover GST fusion proteins produced in *E. coli* cells containing a recombinant pGEX-3X plasmid the GST Gene Fusion System, purchased from Pharmacia Biotech, was used, following the procedure suggested by the manufacturer's instruction manual.

From a glycerol stock stored at -80°C , *E. coli* containing pGEX-3X /GST-Cdc25C (aa 250-56) recombinant was streaked onto a LB medium agar plate in sterility and incubated overnight at 37°C . One colony was picked up and inoculated into 5 ml of LB medium containing the antibiotic of selection then incubated for 12–15 hours at 37°C with vigorous shaking. The grown culture was then diluted 1:100 into fresh pre-warmed LB medium with the antibiotic and let grow at 37°C with shaking. Then the steps followed were exactly the same described in the instruction manual provided with the purification system previously mentioned. Briefly, the bacterial culture was grown until the A_{600} reached 0.5–0.6, then by the addition of 0.1 mM of IPTG to the culture induced to produce fusion protein with a 2–6 hour-incubation. The bacterial culture was centrifuged to sediment the cells and the pellet resuspended in a final volume of ice-cold PBS corresponding to 1/20 of volume of the starting bacterial culture. The cells were disrupted with a sonicator on ice and fusion proteins were solubilised by addition of 1% Triton X-100 to the sonicated cells and gently rotated for 30 minutes. Then the solution was centrifuged and the supernatant was loaded the Glutathione Sepharose 4B and shaken for 2 hours in the cold room. This agarose matrix carried glutathione molecules which bound to the fusion protein through the GST-tag. After washes to remove unbound proteins, the fusion protein was eluted with 10 mM of GSH from the matrix. The

eluted material was monitored for GST-fusions protein by SDS-PAGE. The polyacrylamide gel was subsequently stained with Coomassie Blue R250.

Coomassie Blue R250:

0.25 g Coomassie Blue

45 ml methanol

45 ml glacial acetic acid

to a final volume of 100 ml with deionised water. Filter the solution through paper

The gel was immersed in the coomassie solution for 60 minutes and washed overnight in a de-staining solution (*30 ml methanol, 5 ml glacial acetic acid, to a final volume of 50 ml with deionised water*).

2.13 IN VIVO TUMOR GROWTH

Four week-old female Athymic Swiss NCr-nu/nu mice were obtained from Charles River (Calco, Italy). Mice were maintained under pathogen-free conditions, and provided with food and water ad libitum. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n.116, G.U., suppl.40, 18 febbraio 1992, Circolare No. 8, G.U., 14 luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, Dec 12, 1987; Guide for the use of Laboratory Animals, United States National Research Council, 1996). The clones, (HCT-116 p53 +/+ and p53 -/-) stably transfected with the pSuperior-siRNA Chk1 plasmids, were allowed to grow *in vitro* and then 5×10^6 cells of each clone were implanted subcutaneously in each flank of nude mice. The day

after transplant, mice were randomized to receive or not tetracycline in the drinking water. Tetracycline was provided with drinking water (2 μ g/ml) throughout the period of the experiment and was replaced every other day. The length (L) and the width (W) of the tumor mass were measured by caliper twice weekly, and the tumor volume (TV) was calculated as: $TV = (L \times W^2)/2$ being $W < L$. At the end of the experiment animals were sacrificed and tumor tissue excised. Fragments of the tumors were immediately frozen in liquid nitrogen for protein extraction and other pieces were fixed in formalin 4% for IHC analysis.

2.14 FLOW CYTOMETRIC ANALYSIS OF CELL CYCLE DISTRIBUTION

Cells (about 5×10^5) were fixed 24 after the end of DDP and IR treatments in the siRNA transfection experiments described in chapter 4, while as regarding the experiments described in chapter 5 the cells were fixed at 72 hours after siRNA transfection. Cells were washed twice in ice-cold PBS, fixed in ice-cold 70% ethanol, washed in PBS, resuspended in 2 ml of a solution containing 25 μ g/ml of propidium iodide in PBS and 25 μ l of RNase 1mg/ml in water and stained overnight at 4°C in the dark. Cell cycle analysis was done on at least 10,000 cells for each sample using the FACSCALIBUR instrument (Becton Dickinson, HO, USA). The percentage of cell cycle phase distribution was calculated as previously described (Broggini et al., 1991). In Chapter 4 the G2 abrogation of the cell cycle was calculated following the formula: $1 - \% \text{ of cells in the G2 phase in siRNA transfected treated cells} / \% \text{ of cells in the G2 phase in mock treated cells}$.

2.14.1 CYCLIN B1 LEVELS DETECTION BY FACS ANALYSIS

To detect cyclin B1 levels by FACS, cells were again fixed in ethanol 70% and stored at +4 °C for at least one night. The fixed cells were washed in cold PBS, following permeabilization with 0,25% of Triton X-100 in PBS for 5 minutes on ice. Cells were then washed in PBS and incubated with 200 µl of the anti cyclin B1 antibody (0,5 µg/sample, clone GNS1 from Pharmingen) diluted in rinsing buffer (1% BSA in PBS) overnight at 4 °C by gentle agitation. A blank sample was prepared by incubation of cells with 200 µl of isotype IgG instead of cyclin B1. Cells were then washed once before the incubation with 200 µl of Alexa Fluor 488 F(ab')₂ goat anti mouse IgG (Molecular Probes, Eugene, Oregon, USA) diluted 1:500 in 0,5% (v/v) Tween-20 in PBS for one hour and incubated at room temperature in the dark by gentle agitation. Cells were washed in PBS and subsequently resuspended in a solution containing 5 µg/ml of propidium iodide + 25 µg/ml of RNase A in water to stain the DNA, for one hour at room temperature in the dark. Bi-parametric CyclinB1/DNA flow cytometric analysis was performed on at least 20,000 cells for each sample using the Coulter Epics XL (Beckman Coulter, Miami, FL) and the data analyzed with System II software (Beckman Coulter). The fluorescence pulse was detected using a bandpass filter, 530±30 nm and 620 ± 35 nm for green (cyclinB1) and red (DNA) fluorescence respectively, in combination of a 570 nm dichroic mirror. This was the procedure I used in US to detect cyclinB1 and DNA by FACS. In Mario Negri laboratory, the procedure I followed was similar but to reduce the interference between propidium and FITC emission spectra, the DNA was stained with TO-PRO-3 iodide (TP3). After removing the Alexa 488 the cells were washed in PBS and

stained for 30 minutes at room temperature in the dark with a TP3 solution containing 0.5 μ M TO-PRO-3 in PBS and 25 μ l RNase 1% in PBS and the cell cycle analysis was performed by using a FACSCALIBUR instrument (Beckton Dickinson) equipped by a second red iodide laser emitting at 635 nm and a band pass filter 661 \pm 16 nm for TP3 detection.

2.15 IMMUNOFLUORESCENCE ANALYSIS

To perform immunofluorescence analysis the cells were seeded and grown directly on round glass coverslips in 24 well plates. Depending on the kind of staining performed, the procedure followed is reported below.

2.15.1 DETECTION OF CHK1 IN *p*SUPERIOR DOUBLE INDUCIBLE CLONES

Cells seeded in 24 well plates on coverslips at approximately 15,000 cells/ml of density were subsequently induced or non induced with doxycycline 2 μ g/ml. The inducer was added each 48 hours. The cells either induced or not, after 48,72 and 96 hours from the first induction were washed in PBS once and fixed with 4% paraformaldehyde (PBS solution) for 20 minutes, following two washes in PBS. Cells were permeabilized with Triton X-100 (0.5% in PBS) for 5 minutes and then blocked in Blocking Buffer (2% BSA, 0,2% triton X-100 in PBS) for one hour. The cells were subsequently incubated for one hour with the anti Chk1 primary antibody (clone DCS310 from SIGMA) diluted 1:500 in blocking buffer, following 3 washes in PBS, 5 minutes each. The FITC conjugated secondary antibody Alexa Fluor 488

donkey anti-mouse (Molecular Probes) was added to the cells diluted 1:500 in blocking buffer for one hour in the dark. After 3 washes of 15 minutes each in PBS, cells were incubated with Hoechst 33342 (trihydrochloride, tritydrate, Invitrogen) solution (final concentration: 1 µg/ml) for 20 minutes, following two washes in PBS and one wash in distilled water. The cells were mounted with the mounting media Fluor Save (Calbiochem) observed in a Zeiss Axiophot photomicroscopy equipped for epifluorescence (Carl Zeiss, Oberkochen, Germany) and pictures were taken. If not otherwise specified, both the primary and the secondary antibodies used in the immunofluorescence analysis herein described were diluted in blocking buffer.

2.15.2 MITOTIC PHENOTYPE DETECTION IN HeLa CELLS

To study the mitotic phenotype in HeLa cells different staining and costaining strategies were used.

2.15.2.1 MITOTIC SPINDLE STAINING

HeLa cells transfected with either siRNA scramble or siRNA Chk1 were fixed at 48 and 72 hours post transfection with 4% paraformaldehyde (in PBS solution) for 20 minutes at room temperature, washed in PBS twice and stored at 4 °C until staining. Before staining cells were permeabilized for 5 minutes with Triton X-100 (0.5% in PBS) for 5 minutes then blocked in Blocking Buffer (2% BSA, 0,2% triton X-100 in PBS) for one hour. The cells were then incubated with anti α -tubulin (Harlan, anti rat) diluted 1:100. After over night incubation, cells were washed 3 times in PBS (5 minutes each) and the anti-rat antibody conjugated FITC (from Sigma) was used as

secondary antibody for one hour at room temperature, 1:100 dilution, following 3 washes in PBS (10 minutes each). To stain nuclei, before mounting, cells were incubated with DAPI (final concentration: 30 ng/ml in PBS) for one minute and subsequently mounted with 7 μ l of Vectashield solution (VectorLab). Slides were then observed at the microscope (Zeiss Axioscope, with a Zeiss color camera and the Axiovision software).

Basically the same procedure and the same material were used to observe the mitotic spindle in U2OS cells, but the cells were observed in a Zeiss Axiophot photomicroscopy equipped for epifluorescence (Carl Zeiss, Oberkochen, Germany) as this part was performed in Mario Negri Institute.

2.15.2.2 MITOTIC SPECIFIC CO-STAINING WITH ALPHA TUBULIN AND HISTONE H3 PHOSPHORYLATED AT SERINE 10

To perform the costaining with the two mitotic markers α -tubulin and histone H3 phosphorylated at serine 10, the cells were basically treated exactly as in the previous staining with only α -tubulin and the subsequent staining with the second marker. After the washes from the secondary FITC conjugated anti rat antibody, the slides were incubated with anti histone H3 phosphorylated at serine 10 (anti mouse, Cell signal) diluted 1:50 overnight at 4 °C. Slides were washed 3 times in PBS (5 minutes each) and then incubated with secondary antibody, donkey anti mouse Alexa Fluor 594 (Molecular Probes), diluted 1:800, for one hour at room temperature, following three washes in PBS (10 min. each). DAPI staining and mounting of the slides were performed as previously described.

2.15.2.3 MITOTIC SPINDLE AND CENTROSOMES CO-STAINING

To detect both mitotic spindle and centrosomes in HeLa cells, the following procedure was performed. After the standard fixation with paraformaldehyde 4%, slides were additionally fixed with ice cold methanol for 3 minutes at room temperature, following a PBS wash for 5 minutes. The permeabilization occurred for 30 minutes by gentle shaking at room temperature in 0,5% Triton X-100 (in PBS). The slides were then incubated with anti γ -tubulin (anti mouse, clone GTU-88 from Sigma) diluted 1:6,000 in Blocking Buffer for one hour at room temperature, following three washes in PBS (5 minutes each). The secondary donkey anti mouse Alexa Fluor 594 was then used as secondary antibody (Molecular Probes), at a 1:800 dilution in blocking buffer, following three washes in PBS (15 minutes each). The subsequent incubation with anti α -tubulin and DAPI was performed as previously described.

2.15.3 DAPI-SULFORODAMINE STAINING

U2OS cells seeded on round glass coverslips and transfected with either siRNA scramble or siRNA Chk1 were fixed at 72 hours post transfection with 4% paraformaldehyde (in PBS solution) for 20 minutes at room temperature, then washed in PBS twice and stored at 4 °C until staining. Cells were incubated with a DAPI/sulphorhodamine solution for 15–30 minutes in the dark (*3.5 mg DAPI was dissolved in 100 ml in deionised water; to 0.5 ml of DAPI solution diluted in 100 ml of PBS, 3 mg of sulphorhodamine was added. The volume was brought to 300 ml by adding the Na-Tris HCl pH8 solution:*

Tris base *12 g*
NaCl *6g*
HCl 1 M *54 ml*

H₂O to a final volume of 1 L.

After further washes with deionised water, glass coverslips were mounted in Entellan (Merck, Milan–Italy) and observed in a Zeiss Axiophot photomicroscopy equipped for epifluorescence (Carl Zeiss, Oberkochen, Germany).

2.15.4 LAMIN B STAINING

U2OS cells seeded on round coverslips and transfected with either siRNA scramble or siRNA Chk1 were fixed at 72 hours post transfection with paraformaldehyde 4% and then after usual permeabilization with Triton X-100 0,5% in PBS for 5 minutes and a 1 hour incubation with blocking buffer, were stained with anti lamin B (goat, Santa Cruz Bitechology) diluted 1:500, overnight at 4 °C, following three washes in PBS (5 minutes each). Cells were incubated with FITC conjugated goat secondary antibody (molecular probes) for one hour at room temperature, following three washes in PBS (15 minutes each). DAPI staining and mounting procedures were the same just described for the other immunofluorescence staining.

RESULTS

CHAPTER 3

CHARACTERIZATION OF THE 5' FLANKING REGION OF THE HUMAN CHK1 GENE

3.1 INTRODUCTION

As I largely mentioned in my introduction, following DNA damage a very well organized signal transduction pathway is activated in the cell to prevent genomic instability. The different checkpoint proteins participating in the checkpoint pathways have to be considered as interconnected in exerting their function. In fact, for example, the main player of the G1 checkpoint, p53, also plays a role in regulating the G2-M transition by transactivating some genes involved in the regulation of the Cdk1/CyclinB complex. On the other hand the two main G2 checkpoint regulators, Chk1 and Chk2, play a role in G1 and S phase checkpoint by phosphorylating and leading to degradation of the Cdc25A phosphatase. It has been recently shown by our group and another group (Damia et al., 2001; Gottifredi et al., 2001) how Chk1 and p53 can be interconnected following DNA damage. Chk1 can contribute to the phosphorylation and activation of p53 and at later time points from the introduction of the damage, Chk1 would be downregulated in a p53 dependent manner. This is likely to be the way the cells abrogate the G2 block once the damage has been repaired (figure 3.1). In order to further investigate on the p53-dependent downregulation of Chk1 and more in general to deeply study the way Chk1 is transcriptionally regulated, the human Chk1 gene promoter was isolated and characterized.

3.2 ISOLATION OF THE 5' FLANKING REGION OF THE CHK1 GENE

To isolate the genomic sequences involved in the transcriptional regulation of the Chk1 gene, a human genomic library spotted in duplicate in seven different filters by the UK

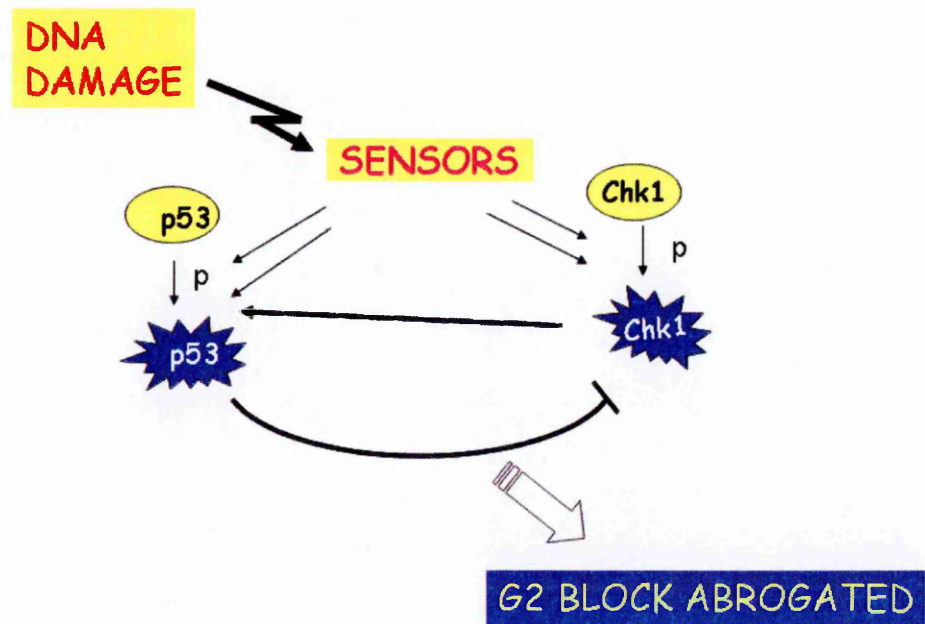


Figure 3.1 p53-Chk1 crosstalk following DNA damage

Schematic representation showing how p53 and Chk1 are interconnected each other after DNA damage. Further explanations in the text.

Human Genome Mapping Project Resource Centre (UK-HGMP-RC) was screened. The filters were screened by using a 5' specific Chk1 cDNA probe to allow the isolation of the promoter region of Chk1. Two positive clones were isolated after the screening which were identified through the coordinates of the filters and then requested to the UK-HGMP-RC (figure 3.2). The two positive clones (53P13 and 253) obtained were grown in LB media and the DNA was isolated from the overnight culture as described in materials and methods. Both the clones contained approximately 100 Kb of DNA, so in order to further characterize the putative Chk1 promoter the first necessary step was to get smaller DNA fragments again probed by the Chk1 gene. A Southern blot analysis, represented in figure 3.3, was then performed on the two clones. The DNA was digested over night with different appropriate restriction enzymes (BamHI, EcoRI, HindII) and half of it was loaded on a 0,8% agarose gel to separate the fragments of different length. At the end of the run the gel was stained with ethidium bromide and the bands visualized by the UV light. The gel was then transferred to nylon filter and hybridized with a small cDNA probe in order to identify the fragments in the genomic sequences of the two positive clones isolated, containing the Chk1 gene. As it can be observed in figure 3.3, panel B, a certain number of digested fragments gave positive hybridization thus containing the sequence of Chk1 used to hybridize the filter. A fragment of approximately 10 Kb, resulted from clone 53P13 after digestion with EcoRI was picked up for the following steps of characterization. First of all this fragment was subcloned in the pBluescript plasmid by the EcoRI site contained in the MCS of this plasmid. The recombinant plasmid obtained permitted the sequencing of the inserted DNA, initially by using the specific plasmid primers contained just near by the MCS, then departing

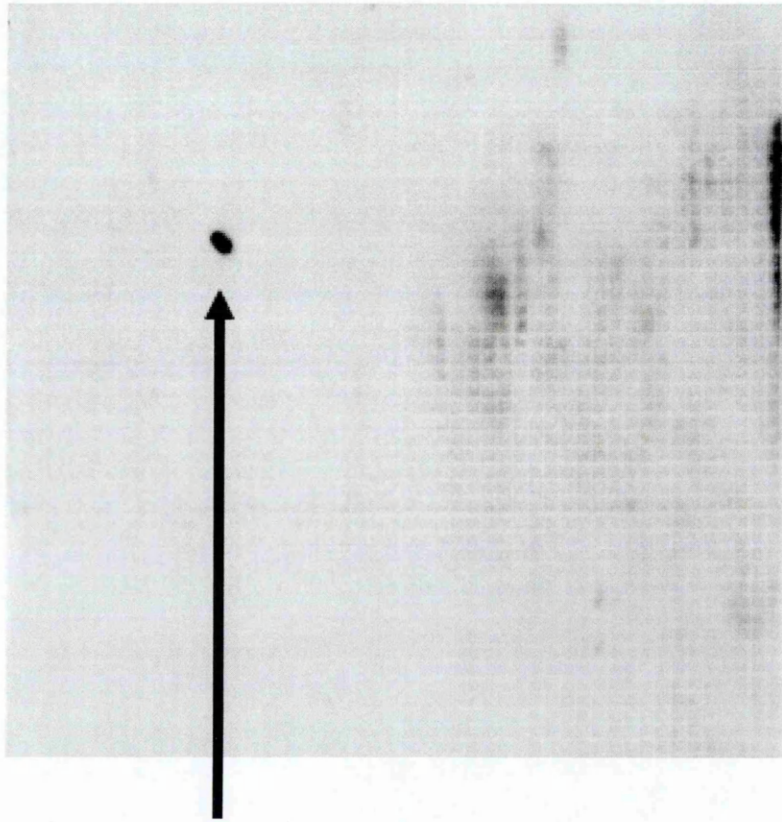


Figure 3.2 Screening of a genomic library

The figure represents an autoradiography resulted from a filter containing genomic DNA probed with a ^{32}P -labeled Chk1 cDNA fragment. Each clone is spotted in double on the filter. The arrow points to one of the two Chk1 positive clones which have been selected from this screening.

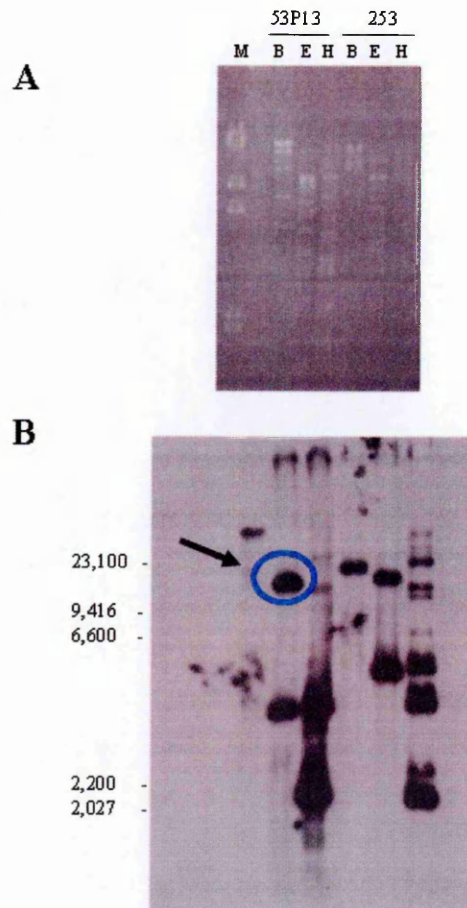


Figure 3.3 Southern blot analysis of two genomic clones isolated from the screening of the genomic library

DNA isolated from the clones was digested with different restriction enzymes and separated on agarose gel.

Panel A. Ethidium bromide staining of the gel. The two clones (53P13 and 253) have been digested with BamHI (B), EcoRI (E) or Hind III (H). M is the molecular weight marker.

Panel B. Autoradiography of the gel transferred to a nylon membrane and hybridized with a ³²P-labelled Chk1 probe. Numbers on the left are derived from molecular weight markers. An arrow indicates the 10kb EcoRI fragment chosen for the following studies.

from the first sequences, other oligonucleotides primers were synthesized and used to further sequence the entire inserted clone. The obtained sequence was then aligned and matched with the human genomic sequences present in the NCBI gene bank database revealing sequence homology to a known sequence (PAC clone no 7622324) and contained the Chk1 second exon, the first intron, the sequence of the first 5' UTR exon, region of the gene transcribed but not translated and a further 5' genomic region as it is represented in figure 3.4.

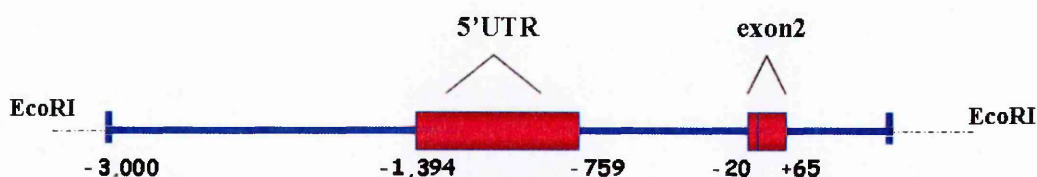


Figure 3.4 5' Chk1 genomic fragment of 10 Kb subcloned in pBluescript

Schematic representation of the genomic Chk1 region comprised in the fragment of 10 Kb previously isolated after the Southern blot Analysis and subsequently subcloned in pBluescript plasmid. The blast of the sequenced fragment with the human genomic sequences deposited in the NCBI gene bank database revealed complete homology with the PAC clone no 7622324 containing the exon 2 (-20;+65), the entire intron 1 (-759;-20), the first untranslated 5' UTR exon (-1,394;-759) and a large 5' genomic region of Chk1 (-3,000;-1,394).

3.3 IDENTIFICATION OF THE CHK1 GENE TRANSCRIPTIONAL STARTING SITES

As it has been already shown that Chk1 generates two transcripts of different length, the presence of two different transcriptional starting sites was hypothesized. I tried to detect the transcriptional starting sites both upstream of the second exon which contain the ATG of the 54 kDa Chk1 protein and upstream of the 5'UTR not translated exon also included in the DNA fragment isolated. I extracted PolyA⁺ RNA from the colon carcinoma cell line HCT-116 which was already ascribed carrying both the Chk1 transcripts (Damia et al., 2001) and two different approaches were at first used to search for the transcriptional starting site: primer extension and race marathon. By primer extension using specific primers located in the second exon, it was possible to locate precisely the starting site at the beginning of the published Chk1 cDNA which was found to be at base -20 from the ATG coding for the first methionine of the Chk1 protein. As it can be observed in figure 3.5, it was possible to count precisely the position of the starting site as a nucleotidic sequence run next to the primer extension reaction. It was possible to calculate the length of the fragment obtained from the primer extension and then predicting the precise transcriptional starting site by counting the nucleotides departing from the known primer used for the specific primer extension reaction (as described in materials and methods). Unfortunately, despite several attempts by using the primer extension strategy and the race marathon strategy, it was not possible to precisely identify the other transcriptional starting site upstream to the 5'UTR region.

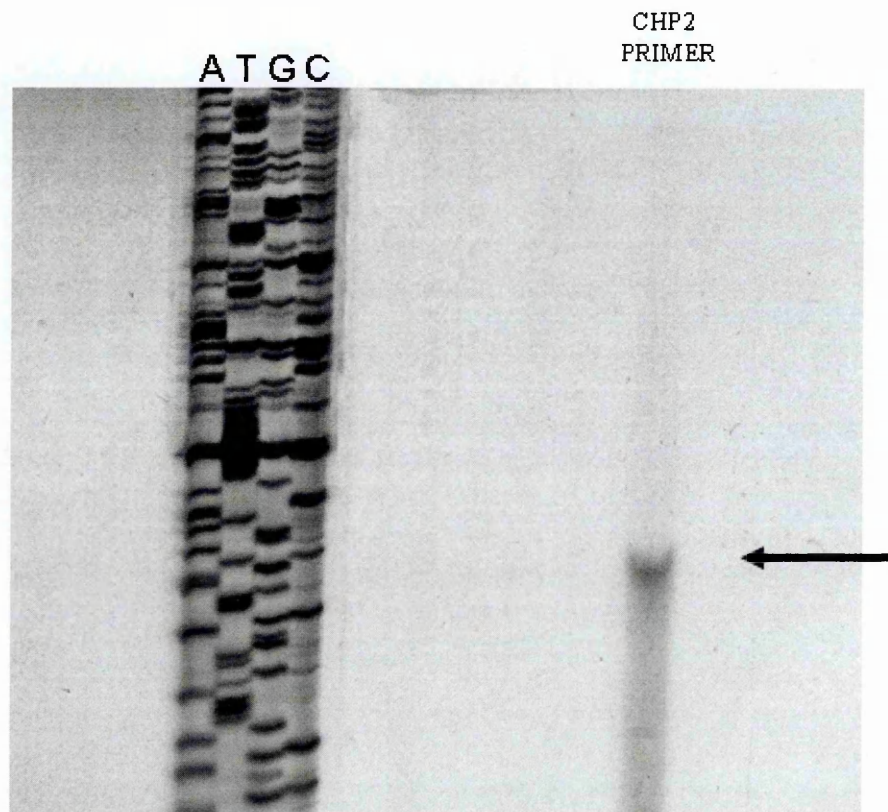


Figure 3.5 Primer extension reaction to detect the transcriptional starting sites of the Chk1 gene

Autoradiography representing the primer extension reaction result by using the primer CHP2 located in the second exon. The arrow points to the band obtained due to the stop of the reaction at the level of the putative transcription initiation site which thanks to the DNA sequence reaction loaded on the gel (left side) was calculated being at -84 bp from the CHP2 primer and at -20 bp from the starting ATG located at the beginning of the second exon.

3.4 PRIMARY STRUCTURE AND FUNCTIONAL ANALYSIS OF THE CHK1 PROMOTER

The 5' Chk1 flanking region between +1 (A of the ATG coding for the first methionine of the Chk1 protein) located in the exon 2, and -2,240 was examined for potential transcription binding sites using MatInspector V2.2 and TFSearch ver 1.3. In figure 3.6 is represented the primary structure of the 5' flanking region of the human Chk1 gene. Regions upstream the first methionine are indicated by negative numbers. The results of the analysis revealed that this putative promoter region did not contain TATA box while four GC boxes could be detected in a region spanning from -1,061 to -590. Several consensus E2F sites as well as potential sites for Sp1, GATA-1,2,3, MZF1 could be detected as shown in the figure 3.6. The GC content in the sequence was determined using an EMBL-EBL program. Whereas the bulk genomic DNA had a GC content of 44%, two CpG islands (defined as areas larger than 200bp that show a GC content of at least 60% and an observed/expected CpG ratio above 0.6) were found located at -1,080 and -698 and at -1,456 and -1,234 from the A coding for the first met of the Chk1 protein. To test the functionality of the isolated genomic sequence, fragments of different length of the genomic DNA isolated containing the 5' region of the Chk1 gene were subcloned in the multiple cloning site of the promoter less pGL2 enhancer vector upstream to the luciferase cDNA. This vector is generally used to determine the efficiency of putative genomic promoter regions to drive the transcription of the luciferase gene. Recombinant clones were selected and the DNAs were partially sequenced to further verify the presence of the right inserts and to determine the

GTGTGGGAT TACACGTGTG ^{Myc} AGCCACCAGG CCCCGGCCTT GAGTAACATT TTATACTTGT TTCACCTTAC TATTATCATA -2161
 ATAGCCAGTT ATGCTTAGTG TTTGTATGTG GGCAGTCTT AAGGACTAAG AAAATATATA TGTATATATA TAAATATAT -2081
 ACATATAAAA ^{HFH2} AAATATGTTT TTTTCTACG GAATCATGCT GTTGCCCGAG CTGGAGTGCA GTAGTGCTCA CTAACCTCTA -2001
 ACTCCTGGAC TCAAACGATT GTTCTGCTTA GGCTCCCAA GTAGCTAGGG CTACAGGGAC ACACCACCAA GCCCGACTAA -1921
 TTCCTAAATT TTTTATTTG TAGAGATGGG ^{GATA} GTCTCCCTAT GTTCCCGAGG CTGGTCTGTA ACTCCTGGCC TCAAGCCATT -1841
 CTCCTGCCCT CGCCTTCCCA AAGTGCTGGA ATTACAGGCA GGAGCCACTG CGCCCGGCCA GTGCTGGAGA ATGTAATGGA -1761
 GATGATCCTG CCTATCCCTG AGTTCCAGTC TAGTTTACTG TCAGAGAATA CTTATTTCGA TTTTCTCTAT TTTGTCTCTG -1681
 AGTCTCTTTC CTCAGAATTI ACTTTTCCAC TTAATGACG TACGCAGCTT TTAATTTGC GTTGTAGAT TTAATTTGGC ^{E2F} -1601
 TCTCCCGCC TGTCTTTTC ACATTAAAA TGAAAAAGTT TGTAGAATA AGCTAAGCAG ATGGTCTTCC TGCAAAAAAGA -1521
 CCGGGCTGAA GTAAAGCATT ^{E2F} CTTTGGAGC TGTTTCACAG AAAAAAGGCA AACTGTGTTA TCCTGACTTC AAGCTCCAAC -1441
 ATAACTGCT CGCTTCTCC GGGAACTTG CCCGCCACA TACACTGAC TGGCTGGCCA GTTCTTTCGA AGCCTCTCGG -1361
 TCCCAACAG GAGTCTCTC CATTTCTCA CATTCGGTC TCAGCAGCTG CTGCTGGTTT CTCGGCTCCA GCACCACGAG -1281
 TACCGCACTC TGAGTTTAC AAAGCACTCT GCCTCAGCA CTGTGATCCT CACAGTCTG TCCGGTGGCC TCACGAGGT -1201
 GCGGTGACG CTTTCAGGC CCAGAGCGGC CAGGAGGAA GCCCGAGCC CGCCTGGAA GCGCAGCGG GTCGTCGCG -1121
 CGCCCTGAG GCTTGGAGG CTGGCTTCC CCTAGCAGG CTGAGGACC GCCAGTCCA GCCTCACACC GGATGCCACT ^{cEts} -1041
 TCATATTGG GCCCAGAGCT CATTCGGCT CGATCGGTC CGCCCTCTT AAATCTCTC AGCCAGGATC TCTCCCGAG -961
 TGCAAGCAG CCTGGGCGG GAGCGGCAAC ATCTCAGT CACCTTTG GAGCGCGA CATTGAGAGG GGCAGGACAC -881
 GGGAGCGGC GCTGTCTTC TTTACGGCG GGTGCGCA GTTTCGCA GCTGAGGCG CTCAAGT TTTGGCGGAAAA ^{E2F} -801
 CGCTGCATT TGGATCTCT CAGTGTGGG CAAAGGACAG TCCGTGAGG AAGGGCGGC GGTAGAGTAG GGAAGGTTTC -721
 TAAAGAAGGA GTTCGGGTC TAGATTAGT AGGAGGGCA TGGTGGGAGA AAGTTAGCAT TGACTAGCGC AGGGGATTGG ^{MZF1} ^{GATA} -641
 AGAGACTCCT GCGAGGGGC GGTTCGGAG GGTGAGGAA TGGTACCAG AGGTTCCCTG TGTGGGGCG GGGGAGGAG ^{SP1} -561
 GGACTAACCC GCTGCTAGT AAGAACCCCA AGAAGAGTC CCAGCCCTTC CTTTCCGCTC TGGGGGCA TTGGGCGCCG ^{E2F} -481
 CCCGCCCTT GGGACGGGT GGGCTGTGA TGGGATGG AATTGGAATT GAGCAATTGG GAGGAGGCG TGCCGAGGC ^{GATA} ^{MZF1} ^{IKAROS} -401
 GTTTCTGCC CCATACCGCT CCATATATCC TCTTCTCTC TTCGCCAGC CCCACCTCT CCTCTCTCT TCCCCAGTCG -321
 TTCGCCGAA AGCATTGTG TCCCACCTCT TCATAACAAC AATTAATTTC CTCTGGGCG TGAGGAGGC AGAATTTCAG ^{E1K1} -241
 CCTTCGGTG GCTTGGAGT GCGGATTGTG ATTTACAGCA CAAATGGTC AGGTTTAGAA TAGTTGTTCG TGGTTGAAAG -161
 ACTTGATAA ATGTGGATGA GATAGAAGG GAAGGCAAGA GCTGTAATT TTCGTGGCA ATCCTGGGA GAGGCGGGG ^{MZF1} ^{MZF1} -81
 TCAGTGGCT CACTGGTAT CTTACACTCT ACATCTTTC TGGATTCCTG CTTTTACAG CCGAGGTGCT CGGTGGAGTC ⁻²⁰ -1
ATGCAGTCCCTTTGTGGAAGACTGGGACTTGGTCAAAACCTGGGAGAAGGTGCCTATGGAGA... Intron 2 +64

Figure 3.6 Primary structure of the 5' flanking region of the human Chk1 gene

The nucleotide of the first methionine codon located in the second exon is numbered +1 while upstream regions are indicated by negative numbers. Outlined sequences represent the 5'UTR and the second exon while the boxes mark the E2F1 binding sites. No TATA regions are identified. Arrows above the sequence indicate consensus sequences for transcription factors.

orientation. As it can be observed in figure 3.7, panel A, the generated constructs simply called A, C, E, H, I, covered the Chk1 genomic region from -2,240 to +78 from the known ATG starting site. The different constructs were then transiently transfected in two different p53 null cell lines: the ovarian cancer cell line SKOV-3 and the osteosarcoma cell line SAOS-2. The luciferase activity assay was then performed 48h after transfection following the procedure described in chapter 2.5. As it is clearly depicted in figure 3.7, panel B, a 25 to 60 fold increase over the activity of the control vector pGL2-enhancer could be detected in all the constructs tested. Moreover the constructs subcloned in an antisense orientation yielded no luciferase activity thus confirming the specificity of the promoter activity of the isolated region (data not shown). Various deletion constructs were also made trying to identify the minimal promoter region in the sequence upstream to both the 5'UTR and the exon2. As depicted in figure 3.8 panel A, shorter fragments were further subcloned both from the original A fragment (into the resulted A1,A2,A3, IR) and from the I fragment (into the so called I1 and I2). As it is possible to observe in figure 3.8, panel B, a substantial decrease in the promoter activity could be detected in the constructs harboring deletions of band A (upstream to the 5'UTR). A comparison of the promoter activity of the different constructs derived from A would suggest the presence of a negative regulatory region located between -1,830 and - 1,762. In fact no luciferase activity was associated with construct A1 while the A2 construct, pretty similar to the A1 construct, but lacking the region above mentioned, shows a significant luciferase activity, although much lower than the original A construct probably due to the lack of the -1,843/1,830 and -1,381/976 parts, with putative positive regulatory activity. As regarding the region upstream to the

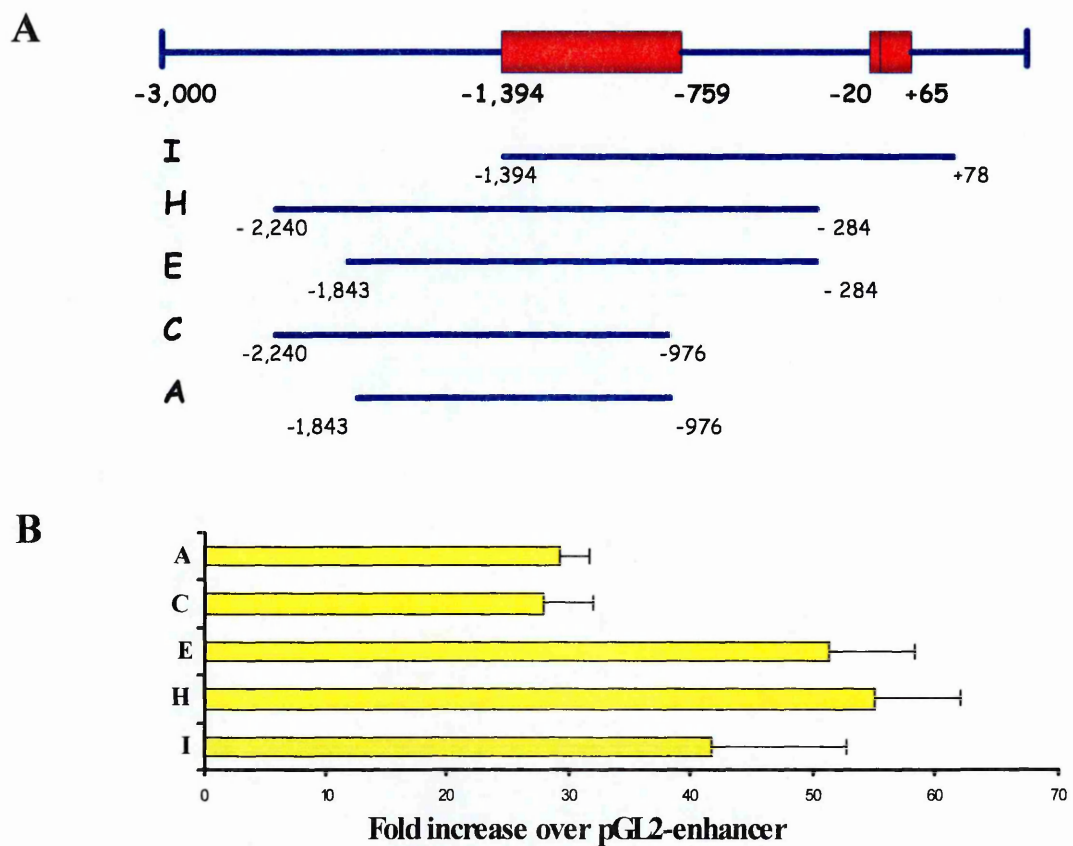


Figure 3.7 Functional characterization of the 5' flanking region of the human Chk1 gene

Panel A. Schematic representation of the different constructs sub-cloned in the reporter plasmid pGL2 enhancer and used in the transient transfection experiments.

Panel B. The fold increase over the pGL2 enhancer vector of luciferase activity is depicted, thus showing the effective promoter activity of the Chk1 genomic region isolated. The results are mean \pm SD of at least three different experiments done in triplicate.

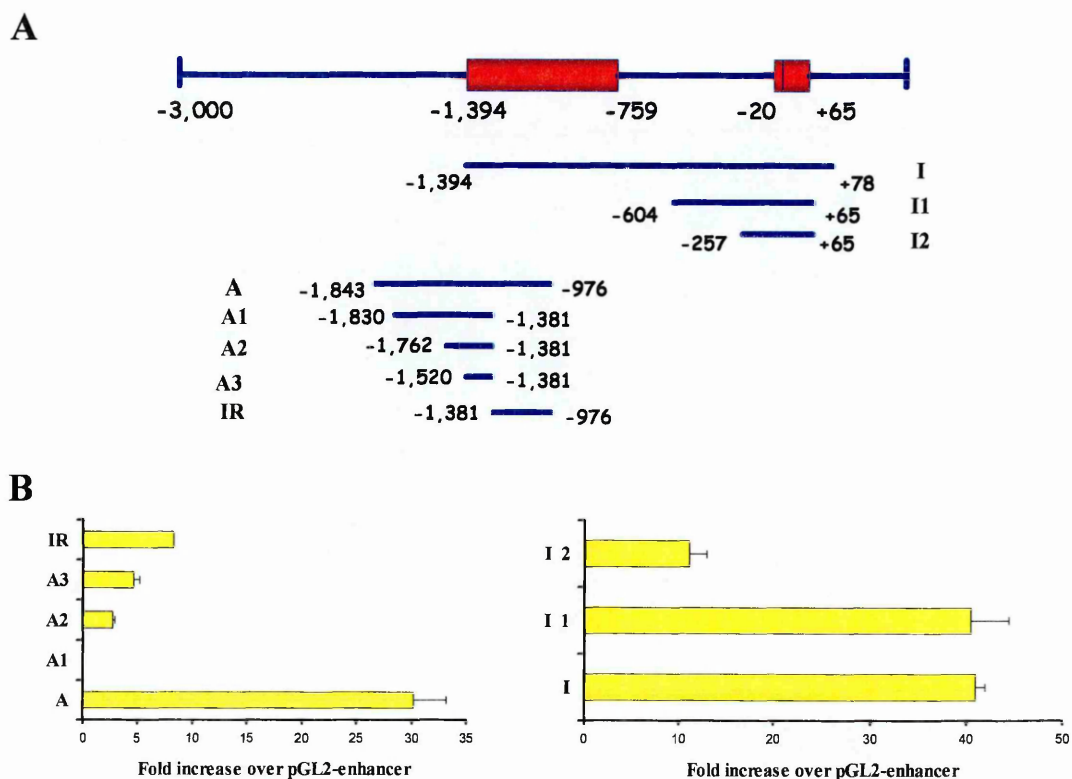


Figure 3.8 Analysis of the minimal promoter activity of the 5' flanking region of the human Chk1 gene

Panel A. Schematic representation of the different constructs subcloned in the reporter vector and used in the transient transfection experiments.

Panel B. Fold increase over the pGL2 enhancer vector luciferase activity.

The results of the luciferase activity assay are normalized by the internal control Renilla and are the mean \pm SD of at least three different experiments done in triplicate

exon 2, a substantial decrease in luciferase activity was observed with the shortest construct I2, while the deletion from -1,394 to -604 did not alter the luciferase activity of the construct I1 thus revealing that most probably the minimal promoter region is represented by the 5' part of the I1 fragment between -604 and -257 as the I2 fragment lacking this region showed a substantial decrease in luciferase activity.

3.5 MODULATION OF THE CHK1 PROMOTER ACTIVITY BY DIFFERENT TRANSCRIPTIONAL FACTORS

Once having identified a functional Chk1 promoter I investigated on the possible modulation of the Chk1 promoter activity by different transcription factors. In particular I focused on p53, p73 (belonging to p53 family) and E2F1.

3.5.1 CHK1 PROMOTER AND p53

At first I focused on the possible negative modulation of the Chk1 promoter by p53 as previous recent data from our laboratory and others have shown a p53 dependent downregulation of Chk1 protein in HCT-116 colon carcinoma cell lines after DNA damaging agents (Damia et al., 2001; Gottifredi et al., 2001). The ability of p53 to cause downregulation of the Chk1 promoter was tested by cotransfecting in the human ovarian cancer cell line SKOV-3, p53 null, an expression vector containing the human p53 cDNA with the different pGL2 promoter constructs whose characterization was just described (pGL2-A,-C,-E,-H). Figure 3.9 shows that in the presence of p53 a decrease in luciferase activity ranging from 30 to 65% was observed in all the constructs tested compared to the luciferase activity observed in the absence of p53. The transcriptional

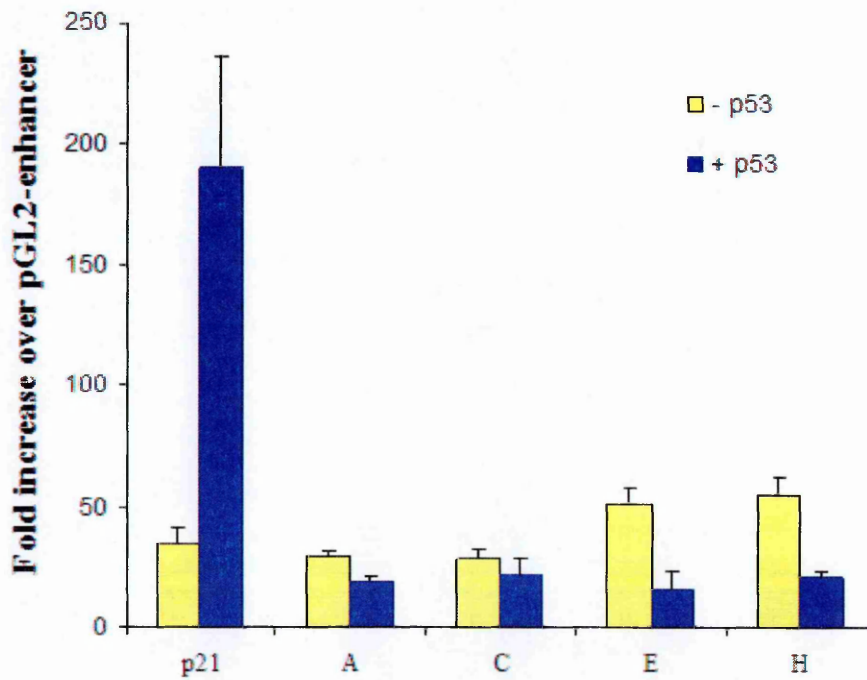


Figure 3.9 p53 modulation of the 5' flanking region of the human Chk1 gene

SKOV-3 cells (p53 null) were transiently transfected with the different promoter constructs either alone (yellow bars) or with the human p53 expressing plasmid (blue bars). The fold increase over the pGL2 enhancer luciferase activity normalized by the internal Renilla control is depicted. As previously underlined these results are mean \pm SD of at least three different experiments done in triplicate.

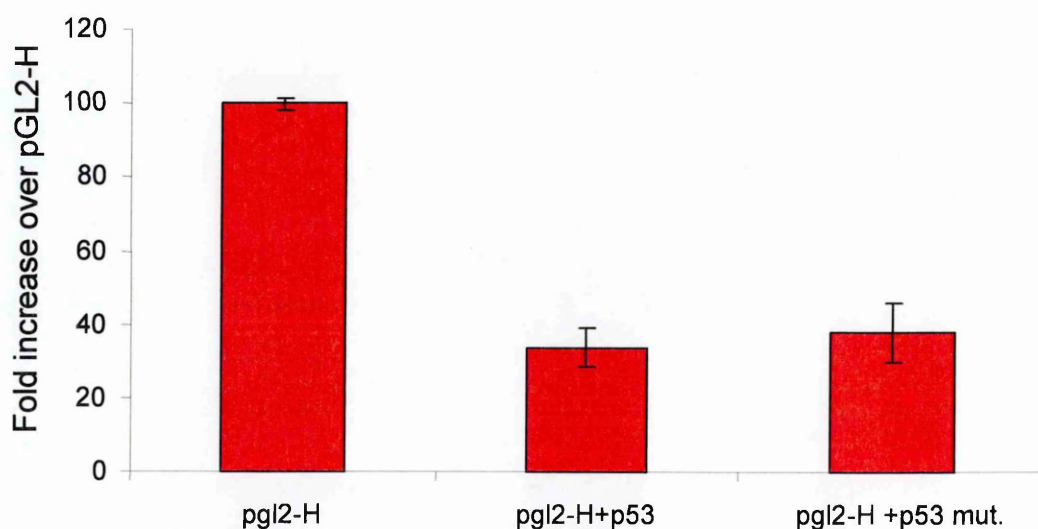


Figure 3.10 Modulation of the 5' flanking region of the human Chk1 gene by p53 either wt or mutated in the DNA binding domain

SKOV-3 cells p53 null were transfected with the pGL2-H construct, the most repressed fragment by p53, alone or in combination with p53 either wt or mutated. The relative luciferase activity over the value obtained by the pGL2-H construct transfection is represented in the figure showing an over 50% decrease in luciferase activity in the presence of both p53 wt and mutated. The results are mean \pm SD of at least three experiments done in triplicate.

repression exerted by p53 on Chk1 promoter fragments appeared more evident if compared with the transcriptional activation induced on the p53 responsive p21 promoter fragment cloned in the pGL2 enhancer vector which represented the positive control of this experiment. The same constructs inserted in an antisense orientation were also co-transfected with the hp53 cDNA and used as negative control of this experiment confirming that their promoter activity (approximately close to 0) was not affected by p53 (data not shown).

The next following experiments were performed by using the pGL2-H construct which was the most repressed fragment by p53. This construct was co-transfected in the same cellular system, the SKOV-3 cell line p53 null, with the expression vector containing either hp53 cDNA wt or its mutated form in the DNA binding domain finding out a similar decrease in luciferase activity (of about 60%) compared to the luciferase activity observed in the absence of p53 (figure 3.10).

This result led to the hypothesis that probably the oncosuppressor p53 does not need to have an intact DNA binding domain to negatively regulate the Chk1 gene but would probably exert its negative regulatory action on the Chk1 promoter through the interaction with additional proteins.

3.5.2 *CHK1 PROMOTER AND THE $\Delta Np73\alpha$ ISOFORM*

On the basis of the just showed p53 dependent downregulation of the Chk1 promoter, I next asked how the ΔN form of p73 which has been previously reported to have a dominant negative function on the p53 transcriptional activity would possibly modulate the Chk1 promoter. The osteosarcoma cell line SAOS-2 was transfected

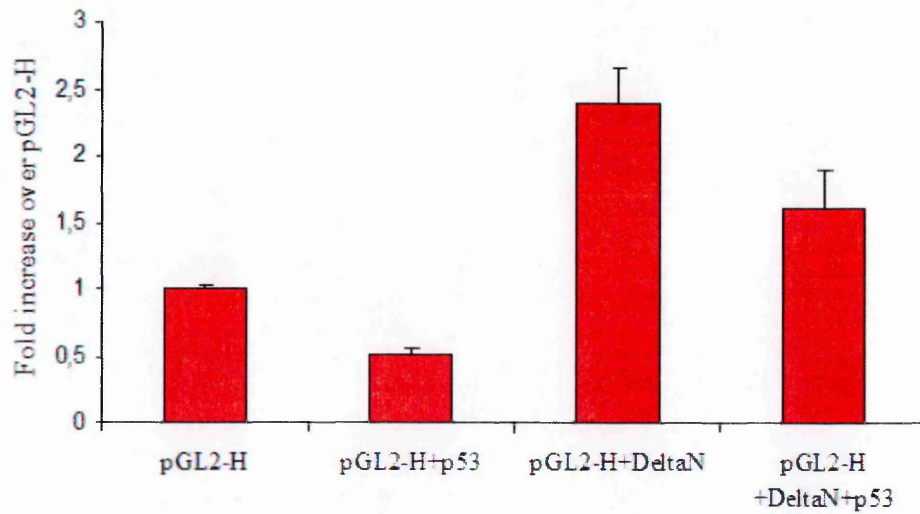


Figure 3.11 ΔN p73 α modulation on the 5' flanking region of the human Chk1 gene
SAOS-2 cells were transfected with the pGL2-H construct alone or in combination with p53, ΔN p73 or both the expression plasmids. The fold increase over the pGL2-H luciferase activity is reported. The results are mean \pm SD of at least three different experiments done in triplicate.

with either the pGL2-H construct alone or with the human Δ Np73 α expression plasmid and as it is possible to observe in figure 3.11, the presence of the p73 isoform induce a slight but reproducible upregulation (approximately 2 fold increase) of the promoter activity of the pGL2-H construct. Furthermore when also the human p53 expression plasmid was cotransfected, no decrease in luciferase activity of the pGL2-H construct was observed thus suggesting that probably the h Δ Np73 α isoform is able to revert the p53 dependent downregulation of Chk1, acting as antagonist on the repressor transcriptional activity of p53.

3.5.3 *CHK1 PROMOTER AND E2F1*

The role of E2F1 in modulating the Chk1 promoter activity was then evaluated as the analysis of the primary structure of the promoter region revealed the presence of six E2F1 binding sites. Moreover indirect evidences recently underlined in literature linked E2F1 to Chk1 (Gottifredi et al., 2001; Ren et al., 2002).

The pGL2-H construct containing the largest number of E2F1 binding sites (represented in figure 3.12) was chosen to perform the co-transfection experiments with the expression vector containing either the hE2F1 wt or the form of hE2F1 (132 mut) mutated in the DNA binding domain. The results obtained by co-transfecting the osteosarcoma cell line SAOS-2 are depicted in figure 3.13 and showed an over 20 fold increase respect to the pGL2-H luciferase activity when hE2F1 wt was co transfected with pGL2-H construct, while in the same experimental setting the co-transfection with the mutant form did not alter the pGL2-H basal luciferase activity. Similar results were obtained in the SKOV-3 cell line and led us to hypothesize that E2F1 has an effective

E2F1 binding sites

-2,240 GTGTTGGGATTACACGTGTGAGCCACCAGGCCCGCCTTGAGTAACATTTTATCTTG
TTTCACTTTACTATTATCATAATAGCCAGTTATGCTTAGTGTGTTGTATGTGGCCAGTC
TTAAGGACTAAGAAAAATATATGTATATATAAAAATATATACATATAAAAAAAT
ATGTTTTTTTTCTACGGAATCATGCTGTTGCCAGGCTGGAGTGCAGTAGTGCTCACTA
ACCTCTAACTCCTGGACTCAAACGATTGTTCTGCTTAGGCCCTCCCAAGTAGCTAGGGCT
ACAGGGACACACCACCAAGCCGACTAATTCTAAATTTTTTTATTTGTAGAGATGGGG
TCTCCCTATGTTCCCCAGGCTGGTCTGAACCTCTGGCCTCAAGCCATTCTCCTGCCCTCG
CCTTCCCAAAGTGCTGGAATTACAGGCAGGAGCCACTGCGCCCGCCAGTGCTGGAGAAT
GTAATGGAGATGATCCTGCCTATCCCTGAGTTCAGTCTAGTTTACTGTCAGAGAATAC
TTATTTCCATTTTCTCTATTTGTCTTGAGTCTCTTTCTCAGAATTTACTTTCCAC
TTAAATGACGTACGCAGCTTTTAAATTTGCGTTGTAAAGATTTATTGCGCTTCCCCGC
CTGTTCTTTGCACATTAAAAATGAAAAAGTTTGTAGAACTAAGCTAAGCAGATGGTCT
TCTGCAAAAAGACCGGGCTGAAGTAAAGCATTGTTTGGAGTTGGTTACAGAAAAA
AGGCAAAACTGGTTATCCTGACTTCAAGCTCCAACATAAAGTCTCGCTTTCTCCGGGA
AACTTGCCCCGCCACATACACTGACTGCGTGCGCAGTTCTTTCGAAGCCTCTCGCTCCC
AACACGGAGTTCTCCATTCTTTCACAGTCCGCTCTCAGCAGCTGCTGCTGGTTTCTCG
GCTCCAGCACCCAGAGTACCGCACTCTGAGGTTTACAAAGCACTCTGCTTCAACGACTGT
GATCCTCACAGTCTGTCCGGTGGCCTCACGCAGGTGGCGGTGCAGCCTTTCAGGCCAG
AGCGGCCAGGAGCGAAGCCCGCAGCCCGCCTGGAAGCGCAGCGCGGTCCGTCCCGCGCCC
CTGAGGCTTGGAGGCTGGGCTTCCCCAGCAGCGCTCGAGCAGCCCACTCGAGCCTCA
CACCGGATGCCACTTCATATTGGGCCAGAGCTCAATTCCCGCGAGTGGCGTCCGCGT
CCTTAAATCTCTCAGCCAGCATCTCTCCCGACTGCAAGCAGCCCTGGGCGGGAGCGG
CAACATCTCCACGTCACCTTTTGGAGTGGCGACATTACAGAGGGGCAGGACACGGGAAC
CGCCCTCTCTTCTTTACGCGCGGGTGGCGAGTTTGGCGCAGCGTGACGCCCTCAAG
TTTGGCGGAAAGCGCGCATTGAGTTGCTGAGTGGTGGGCAAGGACAGTCCGG
TGAGGAAGGGCGGCGGTAGAGTAGGGAAGGTTTCTAAAGAAGGAGTTCCGGGTCTAG
ATTAGTGAGGGAGGGCATGTTGGGAGAAAGTTAGCATTGACTAGCCAGGGGATTGGA
GAGACTCCTGCGGAGGGGCGGTTTGGAGGGTGGAGGAATGGTACCAGGAGGTTCCCGT
TGTGGGGGCGGGGAGGAGGGACTAACCCCTCTAGCTAAGAACCCCAAGGAAGAGT
CCCAGCCCTTCTTTCCGCTCTGGGGCAGTTGGCCCTGCCCCGGCCTTTGGGACGGGTG
GGGCTGTGGATGGGATGGGAATTGGAATTGAGCAATTGGGAGGAGGCGCTGCCGAGG
CGTTTTCTGCCCATACCGCTCCCTATATCCTCTTCTCTTCCCCAGACCCCACTCT
CCCTCCTCCTTCCCCAGTCGTTCCCGGAAAGCATTGTCTCCACCTCTTCATAA -284

Figure 3.12 E2F1 binding sites on H fragment

Nucleotidic sequence of the H fragment which comprises six E2F1 binding sites represented in yellow and circled by the blue boxes. The region in red represents the 5'UTR sequence which is fully included in the H fragment used in the investigation.

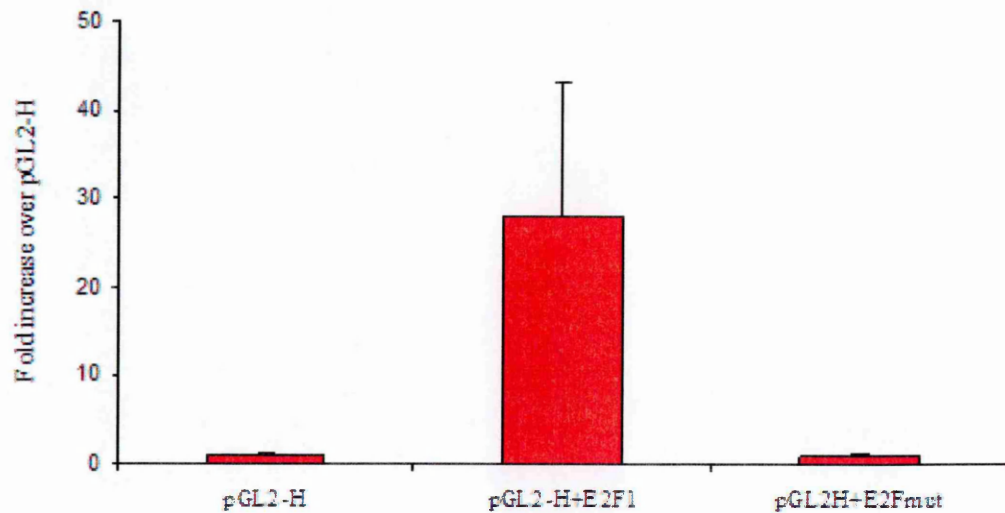


Figure 3.13 E2F1 transcriptional factor modulation on the 5' flanking region of the Chk1 gene

SAOS-2 cells were transfected with the pGL2-H construct alone or in combination with E2F1 expressing plasmid either wt or mutated in the DNA binding domain. The fold increase over the pGL2-H luciferase activity, normalized by the internal Renilla control, is shown. The results are the mean \pm SD of at least three different experiments done in triplicate.

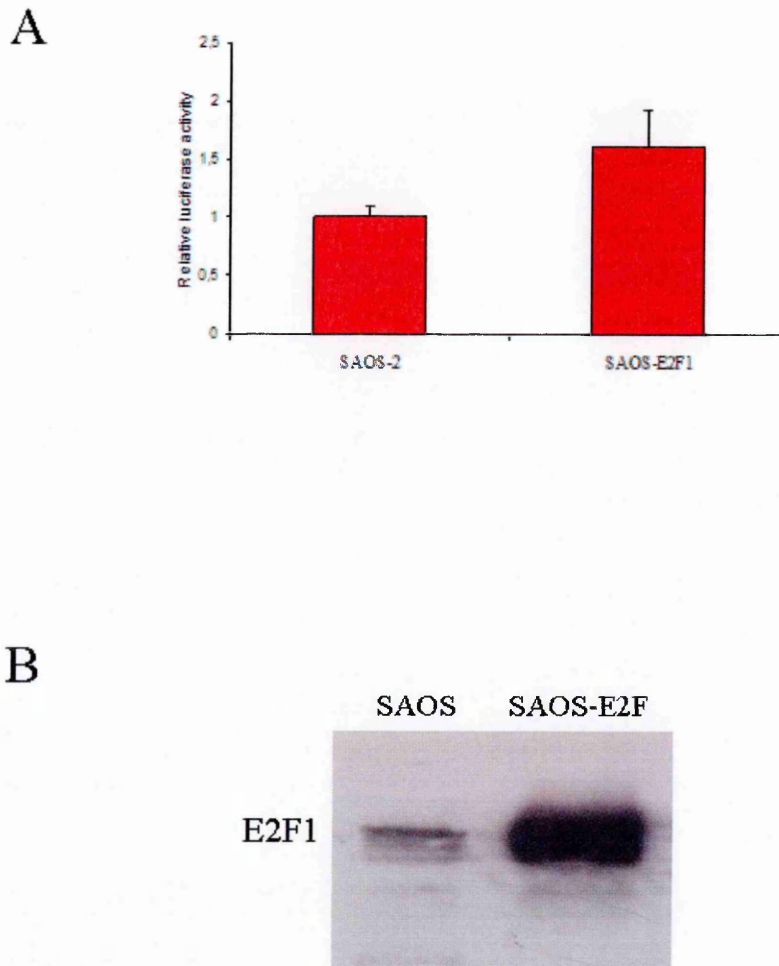


Figure 3.14 E2F1 positive modulation on the Chk1 promoter

Additional confirmation of the positive modulation of the 5' flanking region of the Chk1 gene by the E2F1 transcriptional factor.

Panel A. Luciferase activity of the SAOS-2 cells and of the clone from it over-expressing E2F1 both transfected with the pGL2-H construct. The results are expressed as fold increase over the pGL2-H luciferase activity.

Panel B. Western blot analysis showing hE2F1 expression levels in SAOS-2 and SAOS-E2F over-expressing clone.

role in positively modulating the Chk1 promoter and this positive regulation would most probably require the binding of this transcriptional factor directly on the DNA of the Chk1 promoter. To further corroborate the positive role played by E2F1 on the Chk1 promoter, SAOS-2 cells stably over expressing hE2F1 wt were transfected with the pGL2-H construct. A limited but reproducible increase in luciferase activity could be observed in cells overexpressing hE2F1 as compared to SAOS-2 cells with basal expression of hE2F1 (figure 3.14 panel A). As it can be observed in panel B of the same figure these cells do express higher levels of E2F1 compared to parental cells as the Western blot analysis revealed. It was also possible to study the Chk1 promoter activity on a Retinoblastoma (Rb)-inducible H562 human small cell lung cancer cells (clone 10D5) kindly provided by Dr. G.W. Kristal from Virginia. These cells were stably transfected with a tetracycline inducible expression vector for retinoblastoma protein. As it is possible to show in figure 3.15, a 50% of downregulation of the luciferase activity was observed in Rb-induced H526/10D5 cells when transfected with the pGL2-H construct thus suggesting that the increased Rb protein expression caused the sequestration of the E2F1 protein which can not exert its transcriptional activity on the Chk1 promoter.

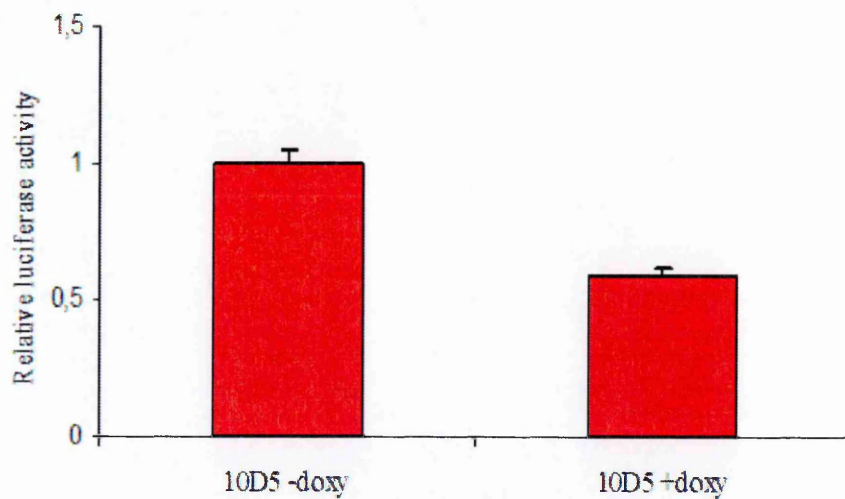


Figure 3.15 Chk1 promoter activity on a Retinoblastoma inducible human cancer cell line

Representation of the relative luciferase activity over the pGL2-H construct on a Retinoblastoma (Rb)-inducible H562 human small cell lung cancer cells (clone 10D5) either induced or non induced with doxycycline (2 μ g/ml) and transfected with the construct studied (pGL2-H). As in all the previous experiments described, the results are mean \pm SD of at least three different experiments done in triplicate.

3.6 DISCUSSION

The investigation conducted in this chapter described for the first time a detailed and specific analysis of the 5' flanking region of Chk1 which was isolated and then structurally and functionally characterized. The 5' region isolated comprised the 3,000 bp upstream to the ATG corresponding to the first methionine translated of the Chk1 protein. It was already previously reported the presence of a 5'UTR (first exon transcribed but not translated) respect to the initially sequenced cDNA (Yolanda Sanchez personal communication) which was also confirmed in the Chk1 5' flanking region isolated and sequenced by our group. The Chk1 gene codes for two different mRNAs (Sanchez et al., 1997) which then generate a single 54kDa polypeptide synthesized starting from the ATG located in the second exon. It was possible to locate by primer extension the transcriptional starting site upstream to the second exon which resulted at -20 from the ATG while I could not unequivocally identified the exact transcriptional starting site upstream the 5' UTR even though I attempted several times by two different techniques (race marathon and primer extension). By applying the molecular cloning strategy, fragments of different length could be subcloned in a reporter plasmid upstream to a luciferase gene and the promoter activity of this region could be confirmed. Two distinct higher promoter activity regions were localized in a region upstream the second coding exon (between -257 and -604 bp from starting ATG) and in a region spanning part of the 5'UTR and its closely upstream region (between -978 and -1843 from starting ATG). This investigation was possible by studying the luciferase activity of different deleted constructs derived from the original longer DNA fragments (see figure 3.8). The Chk1 promoter can be classified as a TATA less

promoter, as by analyzing the primary structure of this region no TATA box near the transcription starting sites could be detected. Beside the accurate structural and functional analysis of the Chk1 promoter, the most interesting aspect of this study was to demonstrate the transcriptional negative regulation of Chk1 by p53 as previous data obtained from our group and another group strongly suggested this hypothesis (Damia et al., 2001; Gottifredi et al., 2001). It was possible to show by co-transfection experiments of hp53 and all the different constructs harboring different fragments of Chk1 promoter region that p53 possibly negatively regulates Chk1 as a 50% decrease in luciferase activity was recorded in the presence of p53. As I already described, p53 is a sequence specific transcription factor that is usually activated in response to different kind of cellular stress, mediating either cell cycle arrest or apoptosis depending on the cell type and the consistence of the damage (Haupt et al., 2002). The mechanisms by which the p53 dependent transcriptional activation occurs have been already described in detail and requires the direct binding of p53 to consensus sequences in the promoter region (El Deiry et al., 1992). On the other hand the p53 dependent transcriptional repression is still poorly described and understood (Murphy et al., 1996). A particular consensus sequence for the putative repressor p53 activity has been previously described (Johnson et al., 2001). This consensus sequence was not detected in the cloned Chk1 promoter region. The p53 mutated in the DNA binding domain did not negatively regulate the Chk1 promoter thus suggesting that, as for other p53 downregulated promoters, p53 effect is not linked to its direct binding to DNA but would most probably occur by the interaction with other additional proteins. Experimental evidence had attributed to the p21/Rb/E2F1 pathway an involvement in the regulation of the repression of the Chk1 promoter

(Gottifredi et al., 2001). Seven different E2F1 binding sites were actually detected on the DNA sequence of Chk1 promoter and it was possible to show that those are functional sites since E2F1 wt but not the form mutated in the DNA binding domain can modulate, upregulating, the Chk1 promoter region. The E2F1 dependent upregulation of the Chk1 promoter can also indirectly explain the p53 dependent downregulation of the Chk1 promoter previously observed following DNA damage. As it is summarized in figure 3.16, in fact, after DNA damage, beside the direct repressor action exerted by p53 on the Chk1 promoter, most probably through the action of other proteins, an additional indirect regulation would also explain the negative influence on the Chk1 promoter. This indirect regulation is more related to the p53 intrinsic ability to transactivate, after DNA damage, the p21 inhibitor which through the direct inhibition of the cdk-cyclins complexes involved in the G1-S transition, would maintain the Rb protein in a hypophosphorylated form thus avoiding the release and activation of the E2F1 transcription factor. The sequestered E2F1 transcription factor would not exert any more its positive action on the Chk1 promoter. The E2F1 positive regulation of the Chk1 promoter could also possibly correlate with the cell cycle specific expression of Chk1 which appears restricted from S to G2-M phases (Kaneko et al., 1999). The expression of E2F target genes are regulated in a cell cycle dependent manner. In G1 phase E2F1 is sequestered by the pRb family members, as just described, and can not exert its transcriptional activity while during the progression of cells from G1 to S phase the G1 cyclin dependent kinases phosphorylate and dissociate the pRb proteins from E2F, resulting in the activation of the genes required for progression into the S phase. The results obtained further supported the evidence that the different players of the

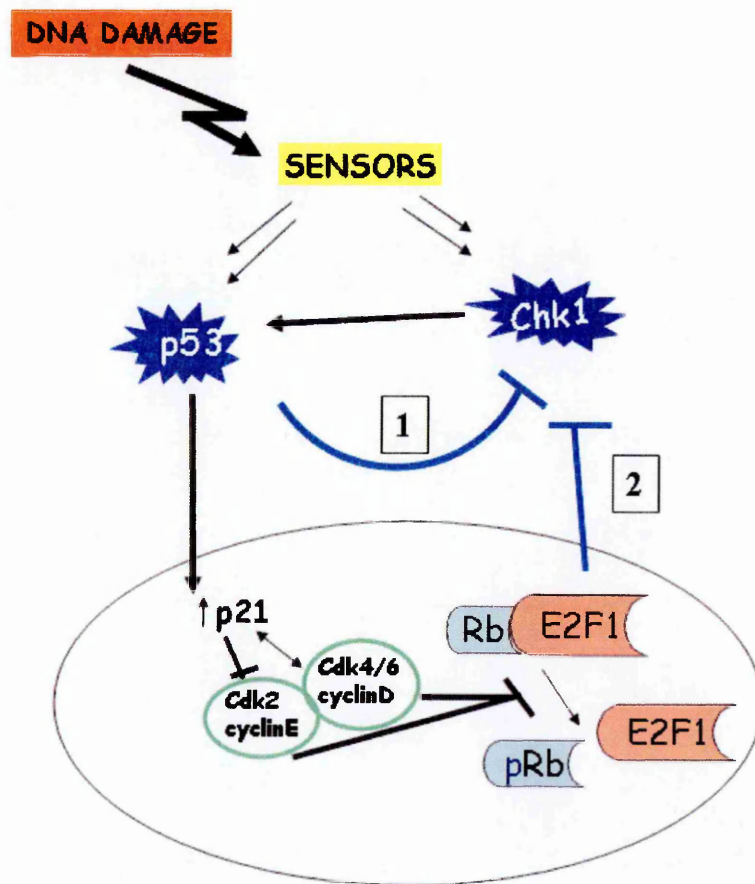


Figure 3.16 p53 negative modulation on the Chk1 promoter after DNA damage

Schematic representation of the possible mechanisms by which p53 negatively regulates the Chk1 promoter after DNA damage.

The first hypothesis (1) would possibly claim the existence of a direct negative action on the Chk1 promoter by p53 by a poorly defined mechanism.

The second hypothesis (2) would suggest an indirect action of p53 on the Chk1 promoter through its ability to transactivate p21 which would lead to the inhibition of the cdk/cyclins complexes involved in the G1-S transition. Thus the Rb factor is maintained in an unphosphorylated form avoiding the E2F1 release which can not exert its positive action on the Chk1 promoter.

checkpoint pathways have to be considered as interconnected each other in exerting their function. By studying the transcriptional regulation of the Chk1 promoter, in fact, it was possible to find out that this gene can be modulated by p53 and E2F1. Furthermore in our experimental system, it was also possible to detect a positive regulation of the Chk1 promoter by the ΔN isoform of p73 α which was ascribed to have a dominant negative action on the p53 activity (Vossio et al., 2002), thus suggesting that upon damage a $\Delta Np73$ upregulation of Chk1 protein levels would help in inducing the G2-M block and the repair of the damage at the early phase followed by a p53 dependent decrease in Chk1 levels that would allow the re-entry of the cell cycle.

CHAPTER 4

ABROGATION OF THE G2 CHECKPOINT BY INHIBITING CHK1 AS POSSIBLE CANCER THERAPY STRATEGY

4.1 INTRODUCTION

As it has been documented in the literature and as my previous observation on the Chk1 transcriptional regulation revealed, an inter-relationship exists among proteins participating in the DNA damage checkpoint pathways and when one of the players of this fine scenario is altered, incidence of tumorigenesis and genetic instability increase. The oncosuppressor p53, which plays a relevant role in the DNA damage checkpoint has been found mutated in a high percentage of human tumors thus inflicting on the functionality of the G1 checkpoint. In these G1 checkpoint defective tumor cells, the sensitivity to different anticancer agents depends primarily on the G2 checkpoint. A strategy to increase the sensitivity to these treatments in tumor cells more than in normal cells would be to try to abrogate the G2 checkpoint. By abrogating the G2 checkpoint, tumor cells, already harboring alterations in the G1 checkpoint pathways would be more sensitive to DNA damaging agents than normal cells and this could lead to an increased therapeutic index. Starting from this assumption I attempted to inhibit the function of the most important player of the G2 checkpoint: the protein kinase Chk1.

In this section I'll describe the different strategies used to try to inhibit Chk1 and the results obtained by using the siRNA strategy which was the most successful approach. To inhibit the Chk1 function the human colon carcinoma cell line HCT116 was chosen as experimental system, as isogenic subclones with a defective G1 checkpoint due to either p53 or p21 inactivated by homologous recombination were available in the laboratory.

4.2 ANTISENSE STRATEGY

Fragments of different length of the available Chk1 cDNA kindly provided by Yolanda Sanchez were isolated by digestion with appropriate restriction enzymes and then sub-cloned in antisense orientation in a mammalian pCMV expression vector. The cloning strategy was used (as already described in materials and methods) to get the recombinant plasmids containing inserts of 198 bp, 675 bp, 1,124 bp correspondent to the different Chk1 cDNA antisense fragments, as it is shown in figure 4.1. The recombinant plasmids were then stably transfected in the human colon carcinoma cell line HCT-116. Single colonies were isolated by seeding the cells at a low density and were selected in the media containing the selection brought by the pcDNA3 plasmid (G418) at the proper concentration. Protein extracts of more than 200 clones were isolated and screened by detecting the Chk1 protein levels by Western blot analysis. No antisense Chk1 clone could be found as all the clones selected presented appreciable levels of Chk1 protein. This negative result was initially attributed to the fundamental role played by Chk1 not only in the G2 checkpoint but also in the physiological growth of the cells. Indeed experimental evidence showed that Chk1 $-/-$ KO mice die early in the embryogenesis. However Zachos et al. have recently shown that the avian DT40 lymphoma cell line Chk1 $-/-$ is viable (Takai et al., 2000; Zachos et al., 2003), so that the effect of the lack of Chk1 in somatic cell lines could be cell line specific and maybe lethal for the experimental system chosen (HCT-116). On the other hand the unsuccessful results could be just related to the intrinsic limitation of this antisense strategy.

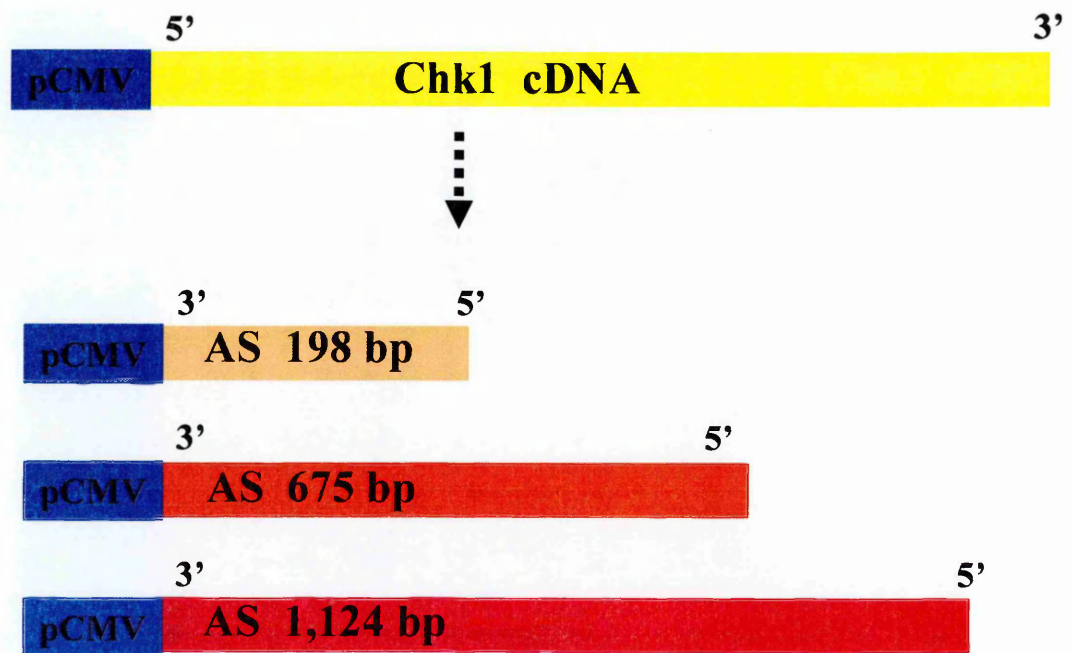


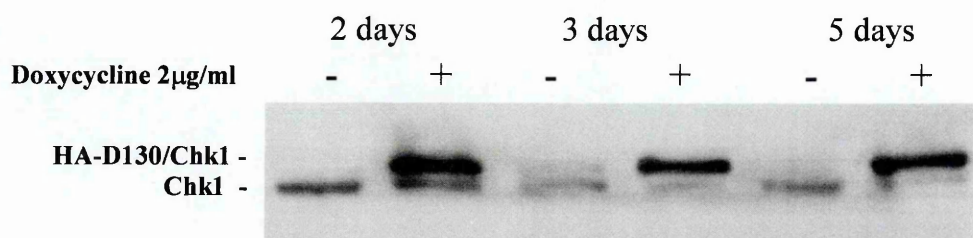
Figure 4.1 Antisense Chk1 constructs

Schematic representation of the Chk1 cDNA fragments of different length subcloned in an antisense orientation in the pcDNA3 expression vector thanks to the availability of the Chk1 cDNA full length expression vector. Briefly, the 198 bp fragment was subcloned between Xho I and BglII restriction sites; the 675 bp fragment was subcloned between XhoI and PvuII and the 1,124 bp fragment was subcloned between XhoI and BamHI as described in materials and methods.

4.3 DOMINANT NEGATIVE PHENOTYPE STRATEGY

The dominant negative phenotype strategy was actually studied in parallel with the antisense strategy in the HCT-116 cell line. A stable and a tetracycline inducible expression system (described in materials and methods) was used in the colon carcinoma cell line HCT-116 to generate a clone with a stable or inducible over-expression of the Chk1 cDNA mutated in aminoacidic position 130 (aspartic acid replaced with the alanine) at the level of the protein kinase domain (D130, dead kinase Chk1). I deeply focused on the isolation and characterization of inducible clones overexpressing the dead kinase Chk1 more than on the stable clones to avoid the problem of the lethal phenotype that the stable altered expression of the Chk1 protein could cause in the cells, as it could be initially hypothesized by unsucces to isolate antisense Chk1 clones. Briefly, the recombinant plasmids were obtained by molecular cloning strategies. The Chk1 dead kinase cDNA fused at 5' to the cDNA correspondent to the HA epitope (coding for a viral protein) was cloned in a plasmid called pcDNA4/TO downstream to a pCMV promoter, responsive to a Tet repressor expressed by a second plasmid, pcDNA6/TR. In the absence of tetracycline in the culture media, the Tet repressor binds to the promoter avoiding the expression of the cDNA of interest. In the presence of tetracycline the Tet repressor detaches and the promoter gets activated expressing the dead kinase Chk1 cDNA. The colon carcinoma cell line HCT-116 Trex 8A, previously obtained in the laboratory by stable transfection with the pcDNA6/TR plasmid was transfected with the pcDNA4/TO HA-D130 plasmid and colonies were isolated as previously described and kept in the appropriate selection media with the two antibiotics whose resistance genes are on the plasmids used. Positive clones were easily detected by Western blot analysis

A



B

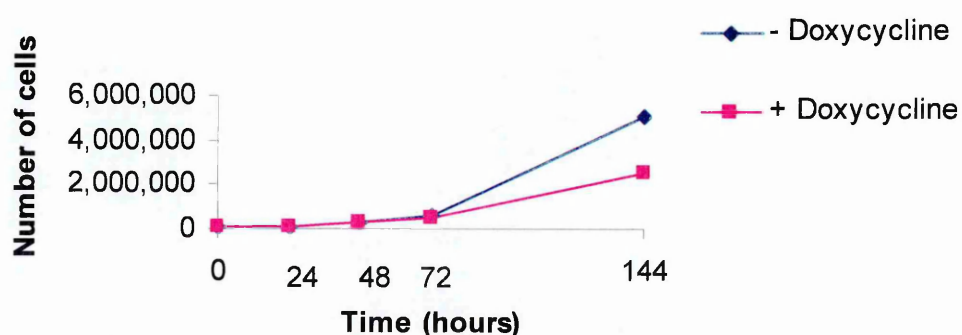


Figure 4.2 Characterization of one of the two positive clones inducibly expressing the dead kinase Chk1

Panel A. Western blot analysis showing the protein levels of both the endogenous Chk1 and the exogenous catalytically inactive Chk1 fused with the HA epitope. The exogenous form resulted at least three times more expressed in the presence of doxycycline (2 µg/ml) in the culture media respect to the endogenous counterpart. The protein levels at 2,3 and 5 days from the first induction are represented. The inducer was added every 48 hours.

Panel B. Cell growth curve of the positive clone either induced or non induced with doxycycline. The cells were counted after 24,48,72 and 144 hours from the first induction. The values represent the mean of three different experiments done in triplicate.

thanks to the molecular weight difference between the endogenous Chk1 protein and the exogenous one fused with the HA epitope. Two tetracycline inducible positive clones were isolated and both showed the same characteristics. I reported for convenience the results regarding just one of the two clones. In figure 4.2 panel A, it is reported the Western blot analysis showing a three times more expression of the catalytically inactive exogenous protein respect to the endogenous one in the presence of doxycycline at a concentration of 2µg/ml (a tetracycline analogue but more stable) in the culture media which was added every 48 hours. As it is possible to observe in figure 4.2 panel B a reduction of cell growth was observed beginning from 72 hours after the continuous induction with doxycycline. This experimental time point was picked up to treat the cells with the anticancer agent cis platinum (DDP) and a clonogenic assay was performed to test the cytotoxic activity of this drug in the presence or in the absence of doxycycline in culture media. As it can be observed in figure 4.3 panel A, no differences in the cytotoxic activity of DDP could be observed in the cells induced and in those non induced by doxycycline. Similar results were obtained treating the cells with different UV doses (figure 4.3, panel B). It was possible to perform the kinase assay on the dead kinase inducible clone thanks to the availability of the Chk1 substrate peptide, (Cdc25C, aa250-256) following the procedure I described in material and methods. As it can be observed in figure 4.4 a residual Chk1 kinase activity after doxycycline induction was present and this could explain the lack of the sensitization of this clone to the DNA damaging agents. The over-expressing exogenous protein can not completely abrogate the kinase activity of the endogenous protein which could actively participate in the G2 checkpoint induced by DNA damage agents such as

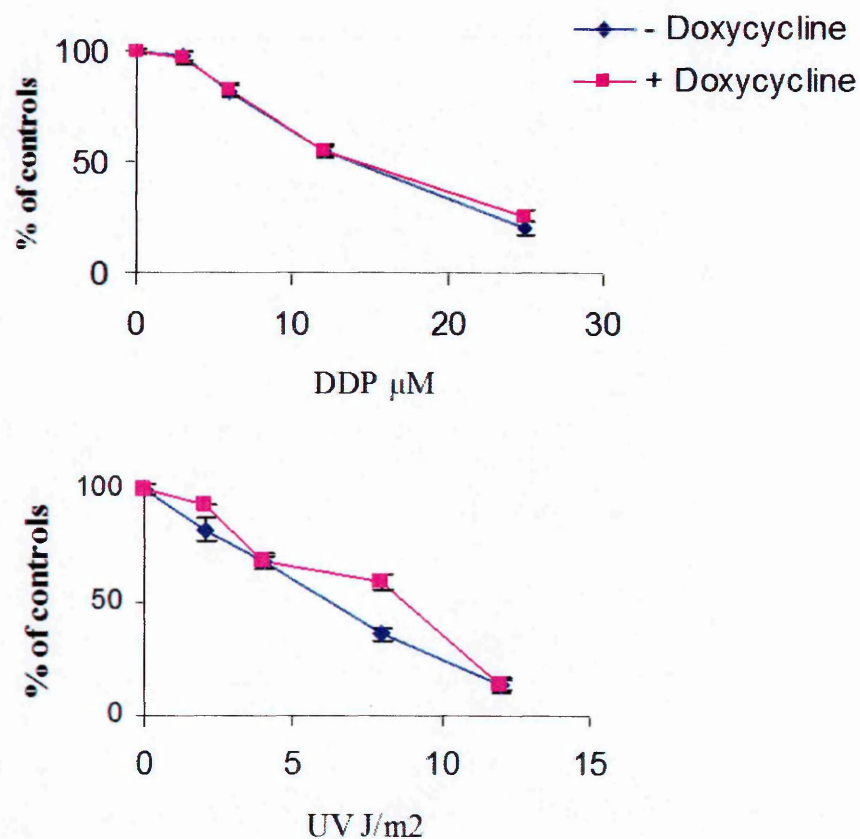


Figure 4.3 Inducible dead kinase Chk1 clone: response to different DNA damaging agents

Cytotoxic activity to DDP (panel A) and to UV (panel B) of the doxycycline inducible dead kinase clone. Cells were seeded in 6 well plates and grown in the absence (blue line) or in the presence (pink line) of 2 $\mu\text{g}/\text{ml}$ of doxycycline. Treatments were performed at the indicated DDP and UV doses. Percentage of cells relative to controls was determined by clonogenic assay. The results are mean \pm SD of at least three different experiments done in triplicate.

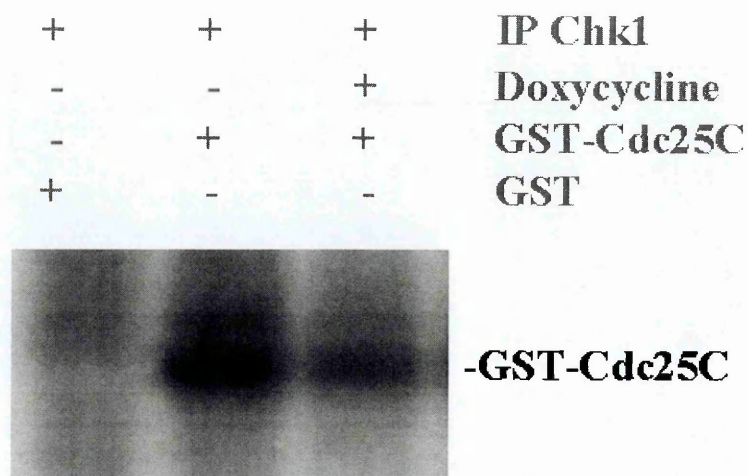


Figure 4.4 Kinase activity of the inducible dead kinase Chk1 clone

Autoradiography of the kinase assay conducted to test the kinase activity of the Chk1 protein in the clone inducibly expressing the dead kinase. The assay was performed as described in chapter 2.12. Briefly, the cellular extracts were immunoprecipitated with the Chk1 antibody and then the recombinant GST-Cdc25C (aa 250-256) was used as a substrate of the reaction in the presence of ^{32}P -ATP and detected by autoradiography after resolving in SDS-PAGE. The first lane represents the negative control incubated with GST only. The second lane represents the substrate phosphorylated by the non induced clone while the third lane outlines the residual substrate phosphorylated by the doxycycline induced clone.

DDP and UV thus possibly explaining the lack of the expected increase in sensitivity to these agents in the clone induced with doxycycline.

4.4 SOMATIC CELLULAR KO STRATEGY

Taking advantage of the technique allowing the targeting of individual genes in human somatic cell lines and the identification and the isolation of the desired homologous recombinants (Sedivy and Dutriaux, 1999; Chan et al., 1999), the colon carcinoma cell line HCT-116, which has been extensively used for the generation of somatic knock out cells for a variety of genes, was picked up to get a somatic isogenic cellular system Chk1^{-/-}. Two recombinant constructs were designed and obtained by the molecular cloning strategy (as I described in materials and methods), thanks to the availability of the genomic DNA previously isolated and further described in the last chapter and containing exons 2 and 3 of the Chk1 gene. Briefly, as it is possible to see in figure 4.5, the two constructs subcloned in the BSSK consisted of a 5' arm containing the 5' flanking region of the gene, the 5'UTR exon 1, intron1, and a 3' arm containing exons 4, 5, introns 3, 4 and part of the intron 5. Exons 2 and 3 and intron 2 were replaced with an antibiotic resistance cassette, either neomycin or hygromycin (as the two alleles should be targeted) whose molecular weight differences (\pm 400bp) with the original genomic sequence rendered possible a PCR screening of the positive clones. To get each of the entire recombinant KO construct multiple step of cloning were necessary. The BSSK-NEO promoter less construct once linearized with an opportune restriction enzyme was stably transfected in the HCT116 cell line and the clones resistant to G418 were screened by PCR analysis taking advantage to the molecular weight difference between

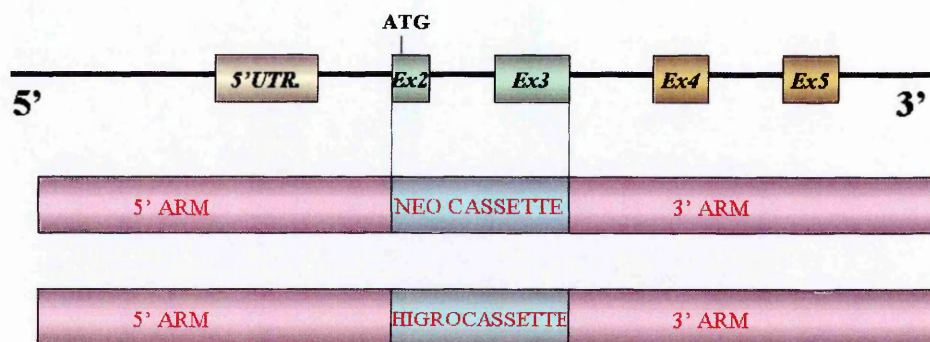


Figure 4.5 KO Chk1 constructs

Schematic representation of the two KO constructs designed and obtained after different molecular cloning steps. The neomycin or hygromycin cassettes were inserted in the construct with the aim to replace the endogenous Exons 2 and 3 of the two alleles by homologous recombination thus creating a Chk1 KO cellular system.

the wt allele and the recombinant allele with the neo cassette present in the case of successful event of homologous recombination. The primers were designed to amplify the region correspondent to the resistant cassette inserted in the Chk1 gene. The positive clones would show two distinct PCR products differing each other around 400 bp. At least 200 clones were screened trying to find the positive one but any homologous recombinant clone could be found. While I was screening the homologous recombinant KO +/- cells, I started to study and evolve in the laboratory the selective and efficient siRNA technique: short double strand RNA molecules which, after having been transfected in the cells, can specifically bind to the mRNA of the gene of interest causing its inactivation and subsequent degradation (as I described in chapter 2.9). After setting up the experimental conditions for siRNA specific inhibition of Chk1 I neglected the time consuming and too complex KO strategy to focus on the siRNA technique strategy to inhibit the Chk1 function.

4.5 siRNA STRATEGY: TRANSIENT SPECIFIC INHIBITION OF CHK1 AND CHK2

I originally designed different siRNAs specific for different Chk1 cDNA targets which were *in vitro* transcribed using the Silencer siRNA construction kit (Ambion) and then screened after transfection in HCT-116 cells by Western blot analysis to verify their functionality. The Chk1 siRNA duplex that more efficiently downregulated the expression of the gene (nucleotides 420-440 from starting ATG of Chk1 cDNA), was picked up and chemically synthesized by Proligo. As I already described in my introduction, together with Chk1, the protein kinase Chk2 also plays a crucial role in the

G2 checkpoint, thus representing another key molecular target to inhibit to increase the sensitivity to anticancer agents in cellular systems with G1 checkpoint defects. I thus decided to use in our experiments both Chk1 and Chk2 siRNA duplexes in order to better define the relative contribution of these two proteins in the G2 checkpoint activation and to provide a relative internal negative control to our specific transient transfections with either Chk1 or Chk2 siRNA duplexes. I chose a validated commercially available Chk2 siRNA duplex (from Xeragon) to perform our experiments. As I previously mentioned I chose as an experimental system the parental HCT-116 cell line with an intact G1 checkpoint and its isogenic sublines with a defective G1 checkpoint due to p53 and p21 gene inactivations. These cell lines were transiently transfected with siRNA Chk1, siRNA Chk2 and with a combination of the two siRNAs. An almost complete suppression of Chk1 and Chk2 expression was observed starting from 24 hours after transfection and persisting up to 96 hours in all the three systems tested. In figure 4.6 for each cell line studied the Western blot analysis showed the Chk1, Chk2 and actin protein levels 48 hours after mock transfection and at 48 and 72 hours after transfection with Chk1, Chk2 or both the specific siRNAs. It is important to note that the downregulation of the proteins of interest was specific as Chk2 levels remain constant after Chk1 downregulation and the same observation is valid for Chk1 after Chk2 downregulation. Actin levels were also detected in each of the cell line studied as additional internal control. In these experimental systems, no modification of cell growth was observed in the absence of DNA damage, not even when both the kinases were knocked down and in the presence or absence of p53 and p21. As it is possible to observe in figure 4.7 the growth of the cells was followed by counting the

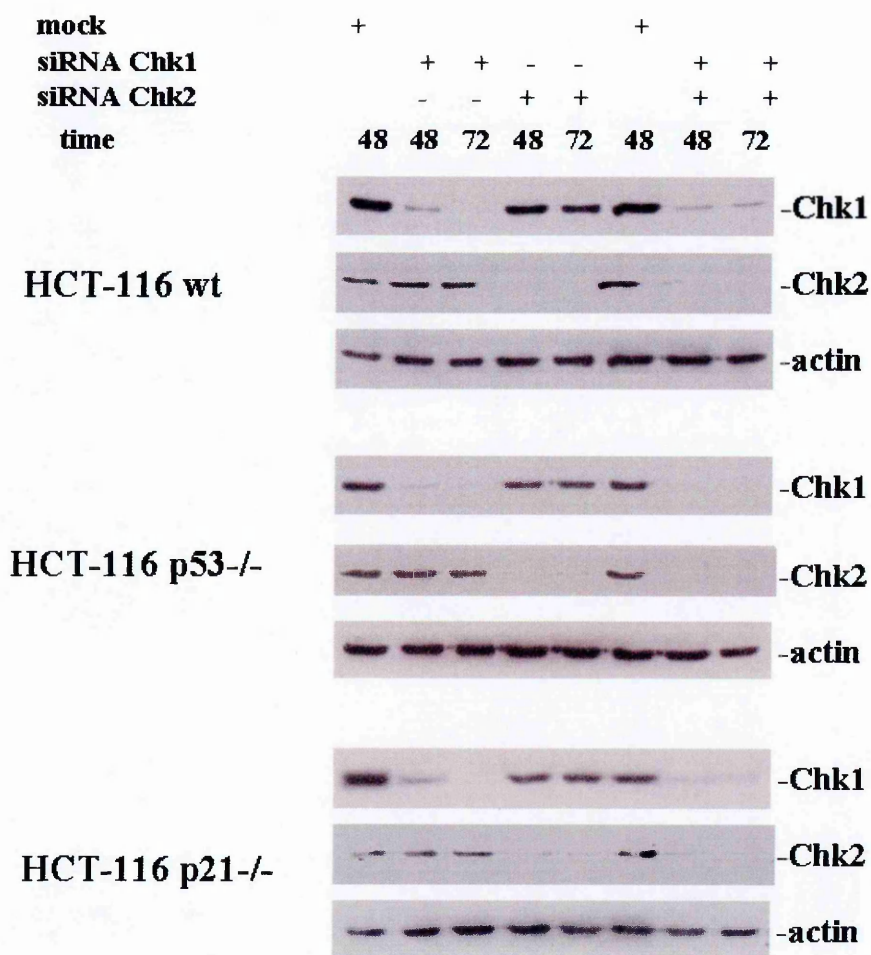


Figure 4.6 Western blot analysis to check for the specific Chk1 and Chk2 inhibition by siRNA

Chk1, Chk2 and actin protein levels were detected by Western blotting analysis in the HCT-116, HCT-116 p53^{-/-} and HCT 116 p21^{-/-} cell lines at different time points after mock transfection (48 hour), or transfection with Chk1, Chk2 or both siRNAs (48 and 72 hour) as described in Material and Methods.

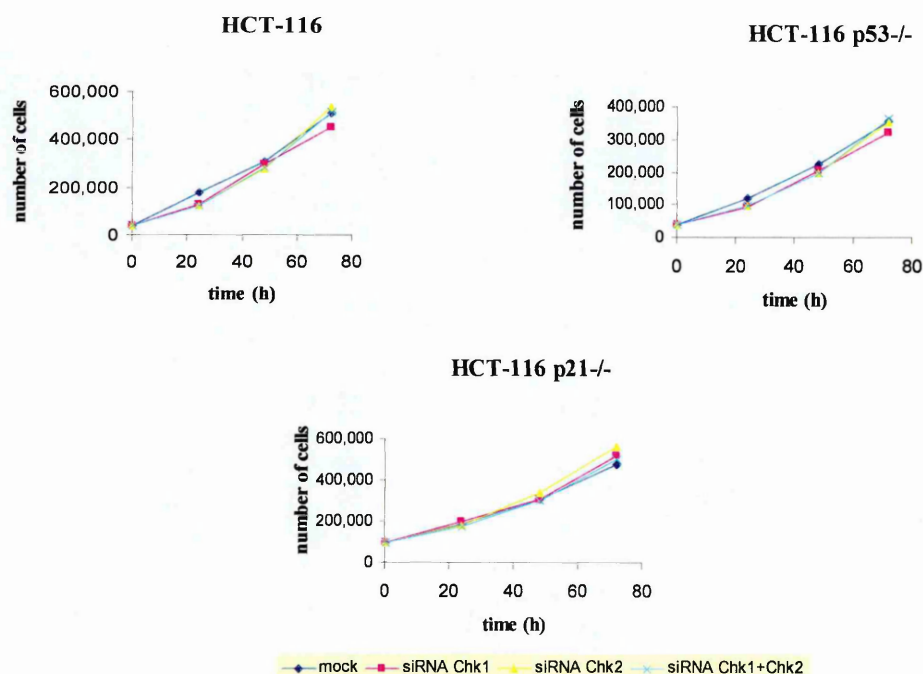


Figure 4.7 Effect of the siRNA Chk1, siRNA Chk2 or both the siRNAs on the growth of the transfected cells

Cell growth curve of the cell lines HCT-116, HCT-116 p53 ^{-/-}, HCT-116 p21 ^{-/-} either untransfected (mock) or transfected with siRNA for Chk1, Chk2 or both the proteins. The yellow box describes the legend. The cells were counted at 24, 48 and 72 hours post siRNA transfection. The values represent the mean of at least three different experiments done in triplicate.

samples from 24 to 72 hours after siRNA transfection. I next asked the question whether the complete downregulation of Chk1, Chk2 or of both the proteins was indeed associated with either a sensitivity of tumor cells to treatment by DNA damaging anticancer agents and/or with an abrogation of the G2-M block induced by the same treatments. DDP and IR were chosen as DNA damaging agents as their mechanism of action is quite clarified, both being able to induce a G2 block of the cell cycle and both being widely used in the clinical practice. For these experiments, 48 hours after transfection with siRNAs (time point at which an almost complete downregulation of Chk1 and Chk2 proteins could be observed) cells were treated with the cross linking agent DDP and IR either to test cell survival by clonogenic assay and to monitor cell cycle perturbation (by FACS analysis) induced by these treatments as described in materials and methods. This investigation has been conducted in parallel in the three cellular lines studied (wt, p53^{-/-}, p21^{-/-}) and for each cell line in mock untransfected cells and in Chk1, Chk2 or both the specific siRNAs transfected cells.

4.5.1 EFFECT OF CHK1, CHK2 AND BOTH PROTEINS DOWNREGULATION ON HCT-116 WT CELL LINE

As depicted in figure 4.8 panel A and C in the mock untransfected cells it is possible to observe a consistent G2 block at 24 hours after the end of the treatment (time point at which previous experiments indicated suitable to see a G2 block), with either DDP or IR conducted at 72 hours post transfection.

A slight reduction of the % of cells in the G2 phase was observable in cells transfected with siRNA Chk1 and with both siRNAs (respectively 44% and 47% respect to 51% in

mock cells) and treated with DDP, leading to approximately a 10% of G2 abrogation. siRNA Chk2 transfection did not modify the % of cells blocked in the G2 phase. An enhanced abrogation of the G2 block was on the contrary observed after IR damage (figure 4.8 panel C); in fact the % of cells blocked in G2 after a dose of 12Gy of IR was 42, 30, and 29 respectively in mock, siRNA Chk1 and siRNA Chk1 and Chk2, corresponding to a 28% of abrogation in siRNA Chk1 and 30% in siRNA Chk1 and Chk2 transfected cells. Again, downregulation of Chk2 did not cause any abrogation of the G2 block. The limited abrogation of the G2 block in parental HCT-116 cells after DDP treatment correlates with the lack of a different sensitivity to DDP treatment in these cells in which Chk1, Chk2 or both proteins were knocked down (figure 4.8 panel B). On the contrary in IR treated cells, a limited but consistent sensitization of about 20% could be detected only in siRNA Chk1 and in siRNA Chk1 and Chk2 transfected cells. (figure 4.8, panel D).

Taken together these data would suggest that Chk1 is not required for DDP cytotoxicity in wt p53 cells, having on the contrary a limited role in the cellular response to IR damage as already described in other cellular systems. Moreover, Chk2 does not seem to have any role in mediating DDP and IR induced G2 block of the cell cycle and in cellular sensitivity to such treatments. As I already described in materials and methods the sensitivity to DDP and IR was studied by performing a clonogenic assay on the cells treated at 48 hours after transfection which were then detached and seeded at low density.

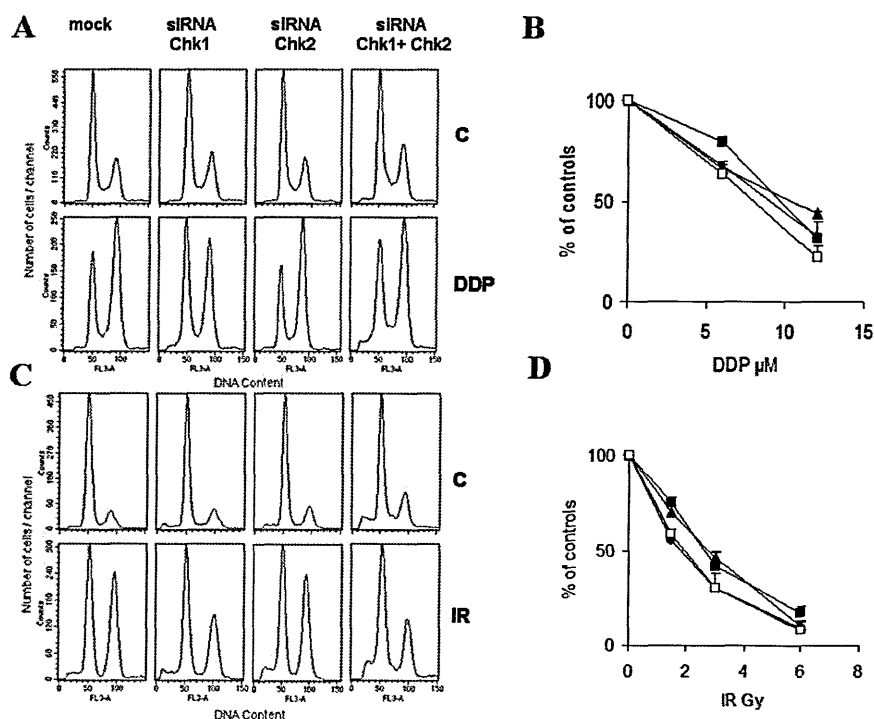


Figure 4.8 Effect of Chk1, Chk2 and both proteins downregulation in the p53 wt HCT 116 cell line after DDP and IR treatments

PANEL A. Cell cycle analysis of mock transfected or siRNAs transfected cells treated or not with DDP 12.5 μ M. The cells were fixed 24 hours after the end of the treatment and processed for cell cycle analysis as described in Materials and Methods.

PANEL B. Cytotoxic effect of DDP in mock transfected cells (■) and in cells transfected with siRNA Chk1 (●), Chk2 (▲) or both the siRNAs (□).

PANEL C. Cell cycle analysis of mock transfected or siRNAs transfected cells treated or not with IR 12 Gy. The cells were fixed 18 hours after the end of IR and processed for cell cycle analysis as described in Materials and Methods.

PANEL D. Cytotoxic effect of IR in mock transfected cells (■) and in cells transfected with siRNA Chk1 (●), Chk2 (▲) or both the siRNAs (□).

4.5.2 EFFECT OF CHK1, CHK2 AND BOTH PROTEIN DOWNREGULATION ON THE HCT-116 CELL LINE p53^{-/-} AND p21^{-/-}

The same experiments were performed in the HCT116 derived cell lines in which p53 and p21 have been inactivated by homologous recombination to better understand the interplay, if any among Chk1, Chk2, p53 and p21 in the cellular response to DDP and IR treatments. As depicted in figure 4.9 panels A and C when p53^{-/-} mock transfected cells were treated with DDP or IR, no G1 block could be observed and the majority of the cells (70%) were blocked in the G2 phase, as expected due to the lack of the p53 checkpoint function. In Chk2 siRNA transfected treated cells the cell cycle profile was similar to the one obtained in mock cells. On the contrary, in Chk1 siRNA and in siRNA Chk1 and Chk2 transfected DDP and IR treated cells, a lower G2 block was observed. The calculated range of G2 abrogation for DDP was between 10 and 20%. This rate of abrogation was associated with a clear shift to the left of the dose response curve following DDP treatment, in cells transfected with only siRNA Chk1 or with both the two siRNAs, (figure 4.9 panel B) with IC₅₀ values changing from 9.9 μ M (in mock transfected cells) to 6.5 μ M and 7 μ M (siRNA Chk1 and siRNA Chk1 and Chk2 transfected cells). Similar results were obtained after IR damage with an even higher degree of abrogation (31-50%) of the IR induced G2 block when the expression of Chk1 was abrogated either alone or in combination with Chk2 (figure 4.9 panel C). Again, the lack of Chk2 did not interfere with the cellular response to the two treatments and the observed effects with the double siRNA transfection are likely to be related to the lack of Chk1 expression (figure 4.9 panel D). These data indicate that in p53^{-/-} background is indeed possible to obtain higher abrogation of the G2 block associated with an increased

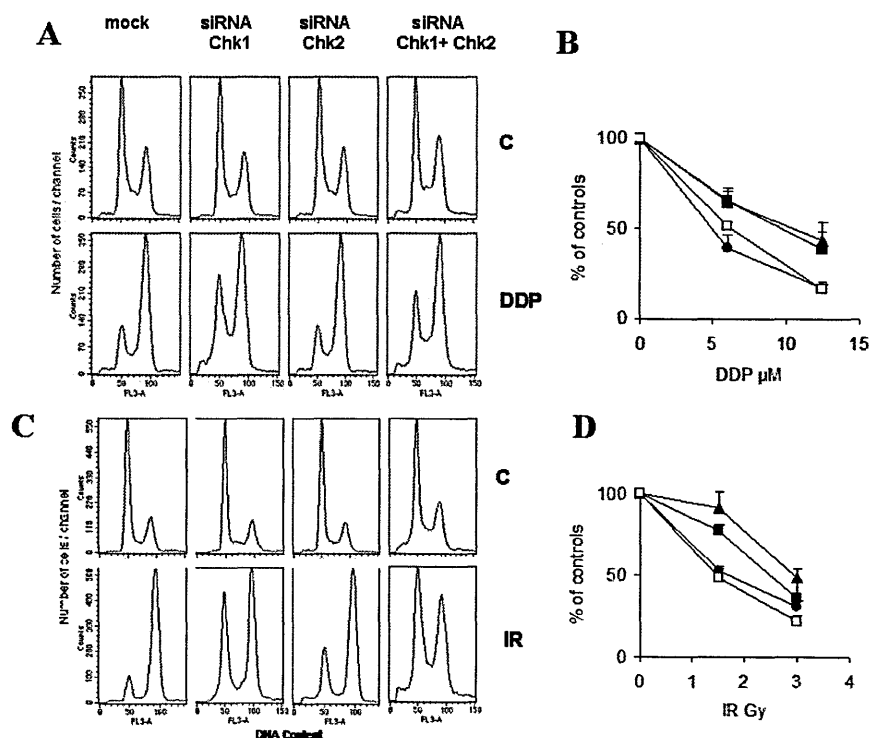


Figure 4.9 Effect of Chk1, Chk2 and both proteins downregulation in the p53-/- HCT 116 cell line after DDP and IR treatments.

PANEL A. Cell cycle analysis of mock transfected or transfected cells treated or not with DDP 12.5 μ M. The cells were fixed 24 hours after the end of the treatment and processed for cell cycle analysis as described in Materials and Methods.

PANEL B. Cytotoxic effect of DDP in mock transfected cells (■) and in cells transfected with siRNA Chk1 (●), Chk2 (▲) or both the siRNAs (□).

PANEL C. Cell cycle analysis of mock transfected or transfected cells treated or not with IR 12 Gy. The cells were fixed 18 hours after the end of IR and processed for cell cycle analysis as described in Materials and Methods.

PANEL D. Cytotoxic effect of IR in mock transfected cells (■) and in cells transfected with siRNA Chk1 (●), Chk2 (▲) or both the siRNAs (□).

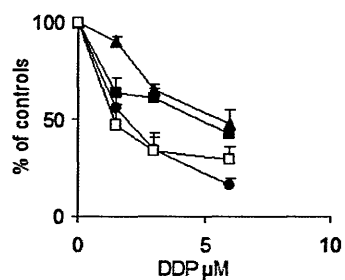
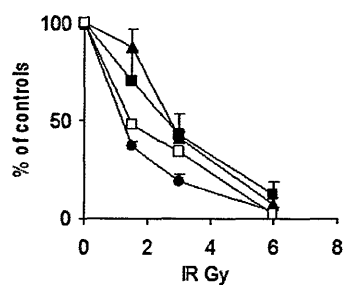
A**B**

Figure 4.10 Effect of Chk1, Chk2 and both proteins downregulation in the p21^{-/-} HCT 116 cell line after DDP and IR treatments

Cytotoxic effect of DDP (panel A) and IR (panel B) in mock transfected cells (■) and in cells transfected with siRNA Chk1 (●), Chk2 (▲) or both the siRNA (□).

sensitivity to treatment with DNA damaging agents than in p53 wt cells. Similar results have been obtained when HCT 116 p21 ^{-/-} cells were used, further indicating that the lack of the G1 checkpoint renders cells more sensitive to G2 abrogation. Figure 4.10 reports the cytotoxic data obtained in HCT 116 p21 ^{-/-} mock, siRNA Chk1, siRNA Chk2 and siRNA Chk1 and Chk2 transfected cells treated with DDP (panel A) and IR (panel B). A clear sensitization to both DDP and IR treatments was observed in siRNA Chk1 and in both siRNAs transfected cells whereas no change in siRNA Chk2 transfected cells again was found.

These data suggested that the inhibition of Chk1 but not of Chk2 caused a greater sensitization to different DNA damaging agents (such as DDP and IR) in the cell lines with a defective G1 checkpoint than in the parental wild type cells. This effect was associated with a G2 abrogation of the cell cycle induced by the same treatment.

4.6 INDUCIBLE siRNA STRATEGY TO DOWNREGULATE CHK1

In order to solve the problem of the transient Chk1 downregulation by the siRNA technique and trying to apply this model in an “*in vivo*” setting an inducible tetracycline system to obtain cellular clones stably expressing the Chk1 siRNA was used. Briefly, as I described in materials and methods the siRNA target was subcloned in the pSuperior plasmid downstream to an H1 promoter which is responsive to the Tet repressor expressed by the second plasmid, pcDNA/TR. In the absence of tetracycline in the culture media, the Tet repressor binds to the promoter avoiding the expression of the siRNA. In the presence of tetracycline the Tet repressor detaches and the H1 promoter gets activated expressing the siRNA of interest at first in a structure of a hairpin which

permits the annealing of the sense and antisense sequences and then leading to the formation of the double strand RNA once the loop is cut by intracellular specific enzymes.

By using this inducible siRNA system it was possible to construct two pSuperior constructs: one containing a single siRNA against Chk1 (21bp target sequence previously used in the transient expression Chk1 experiments) and the other one containing two different siRNA Chk1 target sequences (the already described target sequence and a second one validated by Zhao et al. (2002) with the attempt to further increase the degree and the duration of the inducible Chk1 downregulation.

As discussed in materials and methods to get the double siRNA construct I had to perform additional cloning steps, as I had to include in the original plasmid a second H1 promoter responsive to the Tet repressor.

As internal negative controls to these both single and double siRNA inducible plasmids, the correspondent scramble siRNA target sequences obtained by the proligo website database were also cloned in parallel.

The colon carcinoma HCT-116 p53 $+/+$ and p53 $-/-$ cell lines stably expressing the Tet repressor, were subsequently transfected with the single and double siRNA Chk1 inducible pSuperior plasmids.

The clones were selected and screened as already described in materials and methods and the following sections of this chapter will describe the characterization of these clones which is still an ongoing project.

4.6.1 SINGLE CHK1 siRNA INDUCIBLE EXPRESSION SYSTEM APPROACH

Stably expressing single Chk1 siRNA tetracycline inducible clones were obtained both in the p53 wt and in the p53^{-/-} HCT-116 cell line. By adding doxycycline, a considerable downregulation of the Chk1 protein compared to the non induced cells starting from 48 hours after induction and up to 144 hours could be observed in the selected clones as it is depicted in figure 4.11 panels A and C. As it can be observed in figure 4.11 panels B and D, no differences in cell growth could be found in the presence or absence of the protein of interest in these clones, confirming the results obtained by the siRNA transient system. The effect of Chk1 downregulation on the sensitivity to different anticancer agents such as DDP, etoposide (VP-16), UV rays was then studied. Preliminary data showed an increased activity in p53 deficient cells, doxycycline induced, compared to the p53 wt cells, confirming the data previously obtained with the transient transfection of siRNA. Figure 4.12 represents the results observed after the treatment with etoposide (panel A) and DDP (panel B) after the clonogenic assay performed in the HCT116 p53^{+/+} and p53^{-/-} cell line induced or not with doxycycline. The treatments with the drugs were performed at 72h post continuous induction with doxycycline. As it is possible to observe, the doxycycline induced cells p53^{-/-} appear more sensitive to etoposide and DDP treatment compared to the doxycycline induced p53 wt cells. Figure 5.12 panel C shows the Chk1 and actin protein levels detected by Western blot at the time of the treatment with the drugs, confirming an almost complete suppression of the protein of our interest. To verify if the data obtained *in vitro* could be reproduced in an “*in vivo*” setting, these inducible cellular (p53 wt and p53^{-/-}) clones

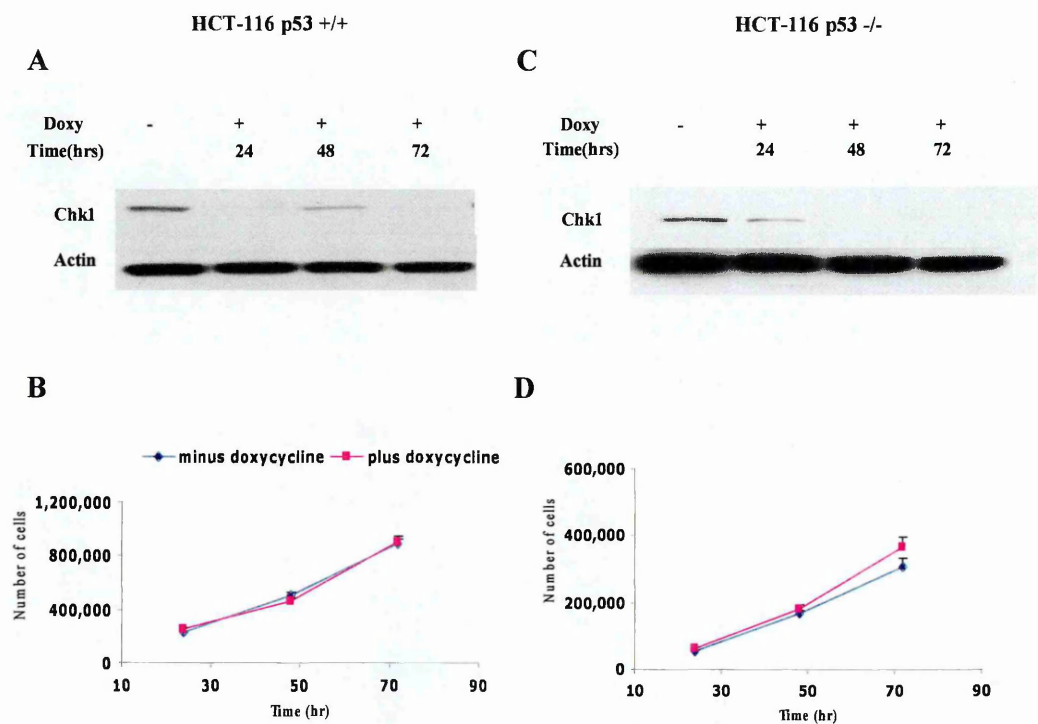


Figure 4.11 Characterization of the doxycycline inducible clones lacking Chk1 obtained in the HCT-116 p53 +/+ and p53 -/-

Panel A and C. Chk1 and actin protein levels were detected by Western blot analysis in the siRNA Chk1 doxycycline inducible clones obtained in the HCT-116 p53 +/+ (A) and HCT-116 p53 -/- (C) at 24,48 and 72 hours post doxycycline induction.

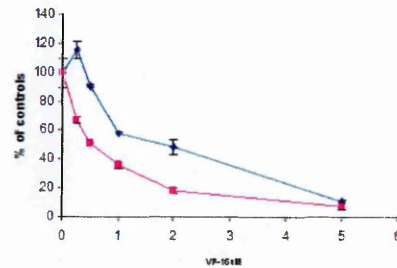
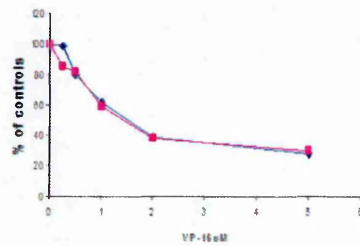
Panel B and D. Cell growth curve of the siRNA Chk1 doxycycline inducible clone obtained in the HCT-116 p53 +/+ (B) and p53-/- (C). The cells were counted at 24, 48, 72 hours post the first doxycycline induction. The blue line represents the non induced cells while the pink line outlines the cells induced with doxycycline. Cells were induced every 48 hours with doxycycline. The values are the mean \pm SD of at least three different experiments done in triplicate.

were transplanted in nude mice. Briefly, the clones, stably transfected with the tetracycline inducible system vectors, were allowed to grow *in vitro* and then 5×10^6 cells of each clone were implanted subcutaneously in each flank of nude mice. The day after transplant, mice were randomized to receive or not tetracycline in the drinking water. Tetracycline was added every other day for all the duration of the experiment. The tumor volume of the induced and non induced mice was measured twice a week and the *in vivo* growth resulted comparable in the presence or in the absence of tetracycline as it is possible to observe in figure 4.13. Autopsies were performed on day 31 after tumor implant and tumors taken. Western blot analysis and immunohistochemical studies are ongoing to evaluate the effective downregulation of Chk1 protein in those tumor tissues derived from mice tetracycline induced. This step of the investigation is actually ongoing as I had to incur in many different technical issues both in setting up IHC for the detection of human Chk1 in transplanted tumors and to find a human specific Chk1 antibody. In fact as regarding the Western blot analysis, the high presence of cellular infiltrates belonging to the mice did not lead to quantify the effective Chk1 downregulation *in vivo*. After having confirmed the functionality of this inducible system in “vivo”, the next step will focus on the chemosensitivity of these tumors transplanted in the animals in the presence or in the absence of tetracycline.

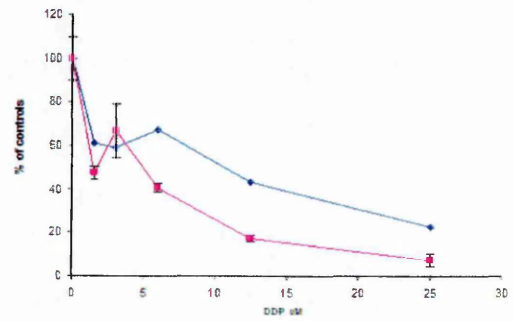
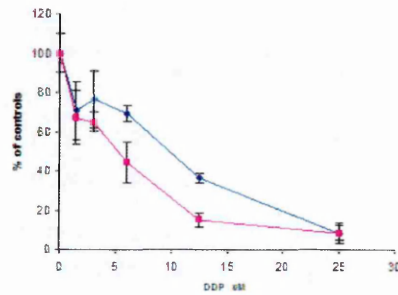
HCT-116 p53 +/+

HCT-116 p53 -/-

A



B



HCT-116

C

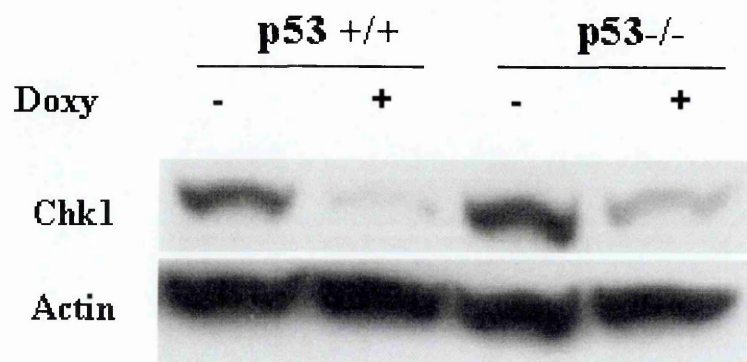


Figure 4.12 Characterization of the doxycycline inducible clones lacking Chk1 obtained in the HCT-116 p53 +/+ and p53 -/-: response to DNA damaging agents

Cytotoxic effect of VP-16 (panel A) and DDP (panel B) in the siRNA Chk1 doxycycline inducible clones derived from HCT-116 p53 +/+ (left part) or from HCT-116 p53 -/- (right part) cells line. The blue line represents the non induced cells while the doxycycline induced cells are indicated with the pink line.

Panel C. Chk1 and actin levels detected by Western blot analysis in the doxycycline inducible clones at the times of the treatments with the drugs to further confirm the inducible downregulation of the Chk1 protein kinase.

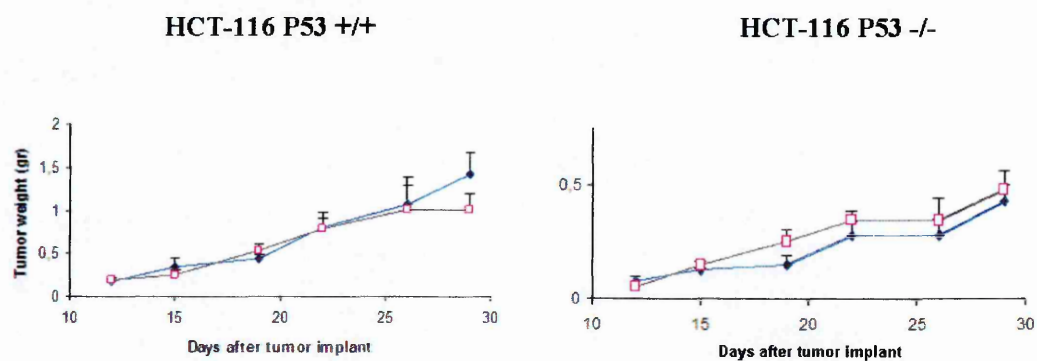


Figure 4.13 *In vivo* growth of the siRNA Chk1 inducible HCT-116 clones

In vivo growth of the HCT-116 clones stably transfected with the pSuperior plasmid inducible expressing the siRNA against Chk1 and transplanted in nude mice. When the tumor was palpable, mice were randomized and either untreated (blue dots) or treated (pink squares) with tetracycline (added in the drinking water at 2 $\mu\text{g/ml}$).

4.6.2. DOUBLE CHK1 siRNA INDUCIBLE EXPRESSION SYSTEM APPROACH

Stably expressing double Chk1 siRNA and double scramble siRNA tetracycline inducible clones were obtained in the p53^{+/+} HCT-116 cell line, while the screening of the clones in the HCT-116 p53^{-/-} cell line is still ongoing. The Western blot analysis conducted on these clones revealed an almost complete downregulation of Chk1 in the doxycycline induced cells starting from 48h up to 144 hours after the induction, while the induced scramble siRNA transfected cells presented the same protein level if compared with the non induced samples (figure 4.14, upper panel). The immunofluorescence analysis was also performed on the paraformaldehyde 4% fixed cells by staining them with a Chk1 specific antibody. As it can be seen in figure 4.15, the cells transfected with the double scramble siRNA plasmid revealed a comparable almost ubiquitary expression of Chk1 in the induced and non induced samples, while a specific decrease in Chk1 expression was clearly detected in the induced double siRNA Chk1 transfected cells thus further confirming the specificity and efficiency of this inducible system. The cell growth curve did not reveal any appreciable difference between the induced and non induced cells (figure 4.14 lower panel). Experiments are ongoing with the aim to test the sensitivity to different anticancer agents in the induced and non induced cells and I am planning to transplant nude mice with the cells inducibly expressing the double siRNA against Chk1 which the results *in vitro* have ascribed to downregulate very well (Western blot analysis and IF results) the protein of our interest.

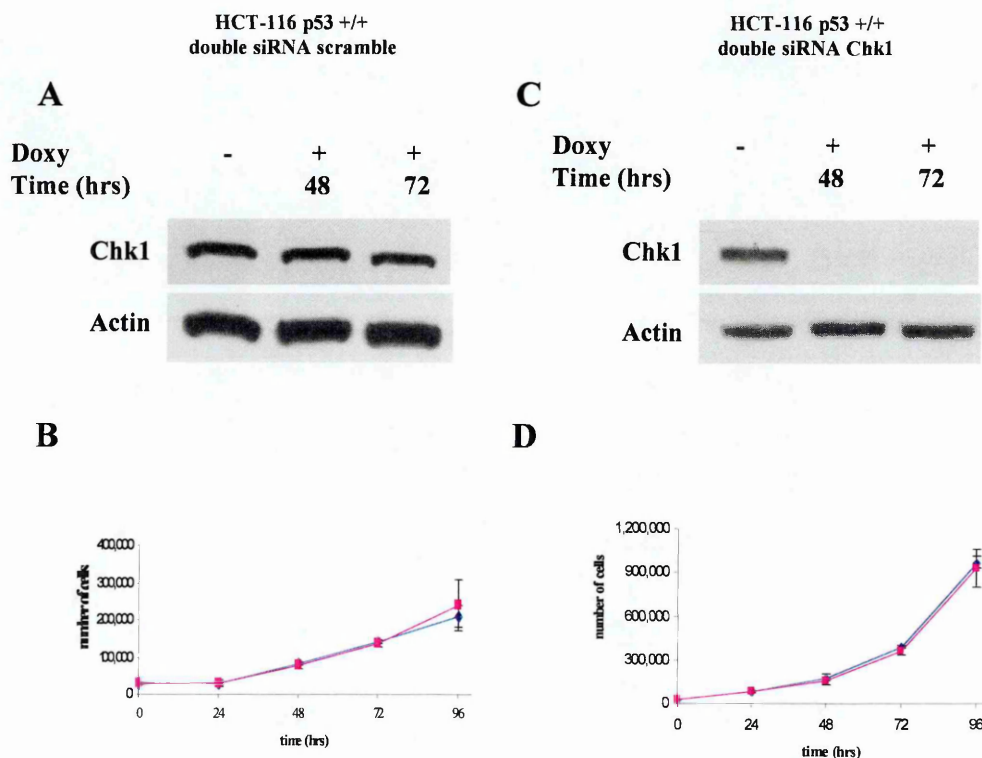


Figure 4.14 Characterization of the double siRNA scramble and siRNA Chk1 inducible HCT 116 +/+ clones

Panel A and C. Chk1 and the internal control actin protein levels by Western blot analysis in the non induced clones or after 48 and 72 hours post induction with doxycycline 2 μ g/ml. Panel B and D. Cell growth curves of the clones either non induced (blue line) or induced (pink line) with doxycycline 2 μ g/ml every 48 hours. The results are mean \pm SD of at least three different experiments done in triplicate.

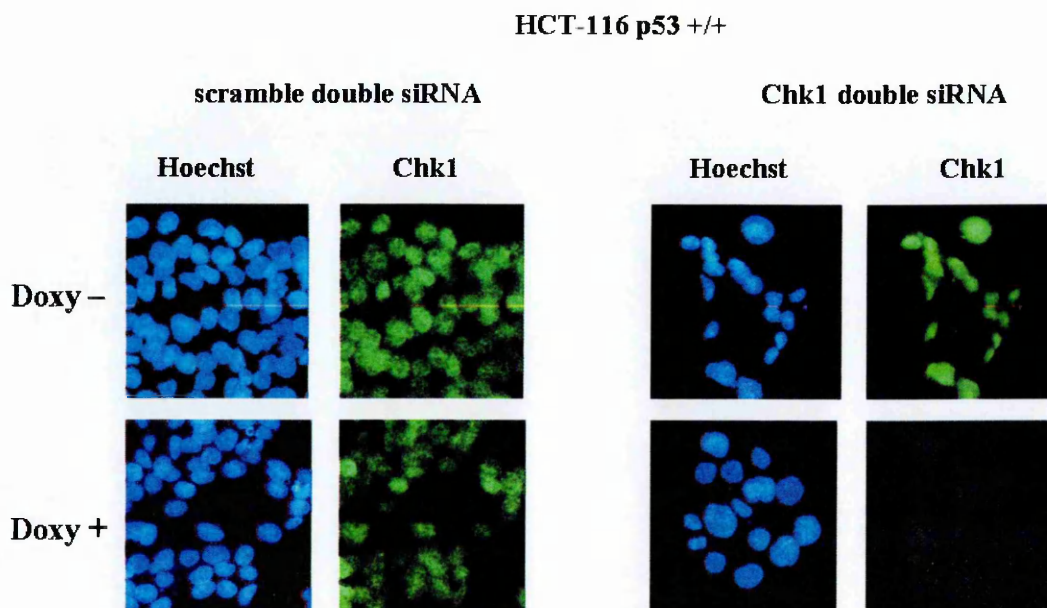


Figure 4.15 Characterization of the double siRNA scramble and siRNA Chk1 inducible HCT 116 +/+ clones: immunofluorescence analysis

Immunofluorescence analysis showing the specific and efficient downregulation of Chk1 in the presence of doxycycline. The cells seeded in 24 well plates on round coverslips were either induced or non every 48 hours with doxycycline and fixed 72 hours after the first induction with 4% paraformaldehyde for 20 minutes. The immunofluorescence analysis was performed as described in materials and methods

4.7 DISCUSSION

The knowledge of the molecular mechanisms at the basis of the DNA damage checkpoint has important implications for both carcinogenesis and cancer therapy. It is becoming clear that tumor cells have checkpoint defects due to mutations of protein with checkpoint function (Liu et al., 2000; Wang et al., 1996). In particular cancer cells have defects in the G1 checkpoint and in this situation the G2 checkpoint acquired particular importance for the repair of the DNA lesions induced by different treatments. By abrogating the residual G2 arrest, it would be possible to specifically sensitize tumor cells to the cytotoxic effect of DNA damaging agents and data from the literature would suggest that this is indeed the case (Donzelli and Draetta, 2003; Luo et al., 2001). Emerging data from the literature have identified Chk1 and Chk2 kinases as key regulators of the G2 checkpoint after DNA damage (Brown et al., 1999). Previous attempts using various strategies to downregulate the kinases of our interest such as the antisense strategy, the dominant negative strategy and KO strategy were unsuccessful and only the selective and efficient siRNA technique (Bunz et al., 1998; Dixon and Norbury, 2002) allowed us to completely down regulate the expression of Chk1, Chk2 and both proteins either in HCT-116 parental cells with an intact G1 checkpoint and in its derived sublines lacking p53 and p21 proteins, thus having a defective G1 checkpoint. Interestingly enough, in our experimental systems the complete abrogation of the kinase proteins did not alter the cell growth in the absence of damage. Having set up the system for a complete abrogation of Chk1 and Chk2 and checked that no cell growth interference was occurring, experiments with two different DNA damaging agents, a cross-linking agent, DDP and IR were performed aimed at investigating their role in the

G2 checkpoint induced by both treatments both by monitoring the G2 perturbation of the cell cycle and by evaluating the cytotoxic effect of the drugs.

The data suggest that Chk2 has no role at all in the cellular response to DDP and IR treatments thus supporting the findings obtained in the HCT-116 Chk2^{-/-} cells where no G1 and G2 checkpoints defects were observed after IR damage (Jallepalli et al., 2003). The fact that, even in a p53 and p21 deficient background, downregulation of Chk2 does not chemosensitize, strongly argues against any important role of Chk2 in mediating the cellular response to DNA damaging agents. However when Chk1 was knocked down clear effects on cell cycle perturbation (a G2 abrogation ranging from 20 to 50%) and cell sensitization to both DDP (in a p53 and p21 deficient background) and IR (in a p53 and p21 deficient background) treatments could indeed be observed (Figures 4.9 and 4.10). Such data clearly reinforce the growing experimental evidence that the G2 abrogation in a G1 checkpoint deficient situation would favor cell death (Donzelli and Draetta, 2003; Luo et al., 2001; Bartek and Lukas, 2003) and outlined the Chk1 protein kinase as an important target to inhibit to improve the efficacy of anticancer agents in human tumors with a defective G1 checkpoint. The siRNA Chk1 inducible tetracycline system was chosen to solve the problem of the transient nature of the synthetic siRNAs and most of all to apply this model in an '*in vivo*' setting. Preliminary data obtained with stable cellular clones inducibly expressing one or two different siRNAs Chk1 further confirmed the data obtained with the synthetic siRNAs as tetracycline induced p53^{-/-} clones showed an increase sensitivity to different anticancer agents. Experiments are ongoing to verify the functionality of this system *in vivo*. As for the siRNA transient system, I could not detect any relevant difference in cell growth in the stable clones

tetracycline inducing the siRNA Chk1 if compared with the non induced clones. While Chk1 has been shown to be essential for mammalian development and viability as suggested by the early embryonic lethality in Chk1 deficient mice and the acute lethality of Chk1 deficient embryonic cells (Takai et al., 2000), Chk1 ^{-/-} DT40 somatic cells obtained by homologous recombination could proliferate, even if at a slower rate due to an increased apoptotic cell death (Zachos et al., 2003). In our experimental system no modification of cell growth could be observed, not even when both kinases have been knocked down and in the presence or absence of p53 and p21, leading to the initial hypothesis that probably this protein is not essential for the human somatic cell lines. This aspect is actually still far to be completely understood and will be the subject of my next chapter.

CHAPTER 5

INVESTIGATION OF THE PHYSIOLOGICAL ROLE OF THE PROTEIN KINASE CHK1 IN DIFFERENT SOMATIC CANCER CELL LINES

5.1 INTRODUCTION

Although a fundamental role in embryogenesis has been attributed to Chk1 in the past (Chk1 $-/-$ mice die at E6.5 days), its role in human somatic cell lines still appears controversial. As I described in paragraph 1.7.1.2 of my introduction in the last 2-3 years different groups have focused their studies on this aspect. Gillespie and his group with the isolation and the characterization of the somatic lymphoma DT40 Chk1 $-/-$ avian cell line provided evidence that Chk1 is not essential for the survival of this cell line (Zachos et al., 2003). These data corroborated the data obtained in the colon carcinoma cell line HCT116, where the down-regulation of Chk1 by siRNA did have no effect on cell growth. During the time I spent in the laboratory directed by Yolanda Sanchez at Cincinnati University, US, I mainly focused on the physiological role of Chk1 starting from the initial observation of a possible inter-relationship existing between Chk1 and two proteins (centrin2 and NTKL) involved in centrosome biology (Kato et al., 2002; Salisbury et al., 2002). The project starting points were the results previously obtained in Sanchez's laboratory, from a binding screening of a human HeLa cDNA expression library using as a probe a phosphorylated human Chk1 isolated from insect cells, where two proteins involved in centrosomes biology, centrin2 and NTKL, were identified as interacting with GST-pChk1. Preliminary data confirmed this inter-relationship as a kinase assay evidenced a clear "*in vitro*" phosphorylation of NTKL variant 1 by GST-Chk1, while a GST pull down assay showed the existence of an interaction between centrin-2 and Chk1 (data not shown). The characterization and the biological significance of these inter-relationships are still under investigation being part of an ongoing project but also represented a starting point for the last part of my investigation

that I'm including in the thesis with the aim to better understand the effective physiological role played by Chk1 in human somatic cancer cell lines.

5.2 CHK1 AND THE MITOTIC PHENOTYPE

Beside the putative inter relationship between Chk1 and two centrosomes related proteins (Sanchez lab data), recently data published by Bartek J. and his group, did attribute to Chk1 a role in the biology of the centrosomes. As I already described in paragraph 1.7.1.2.1 of my introduction, Chk1 protein kinase was observed to be present not only in the nucleus but also in the centrosomes of interphase cells (Kramer et al., 2004). According to this study, Chk1 would dissociate from centrosomes at the onset of prophase concomitant with centrosomes separation. The centrosomes play an important role in the spindle formation during mitosis, important for segregation of chromosomes and for a faithful transmission of the entire genome to the daughter cells. Defects in the spindle formation could bring to aberrant chromosomal segregation and to a change in chromosomal number and structure. In order to investigate the role, if any, of Chk1 in determining a correct mitotic phenotype, I applied the siRNA technique to downregulate the Chk1 protein in the cervical cancer cell line HeLa/E6, whose cDNA was previously used in the binding screening investigation just cited. The same transfection procedure and conditions that I set up for the previous part of my work in HCT-116 cells were also used in HeLa cells but, with the aim to increase the downregulation of the protein of interest I designed the double strand siRNA by introducing an asymmetry in the sequence as Zamore and his group recently described (Schwarz et al., 2003). I again ordered the duplex from Proligo and I used the same

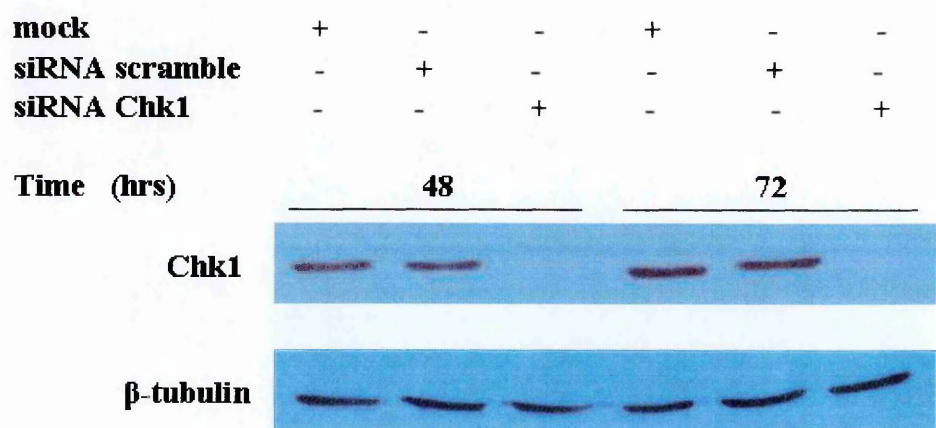


Figure 5.1 Western blot analysis in HeLa cells to test the Chk1 downregulation by siRNA

Chk1 and β -tubulin protein levels were detected by Western Blotting Analysis in HeLa cells at 48 and 72 hours after mock transfection or transfection with either scramble or Chk1 siRNA presenting an asymmetry in its sequence as described in materials and methods.

previous concentration (60nM) to transfect HeLa cells as I did for HCT-116 cell line. I also designed the correspondent siRNA scramble of the Chk1 target sequence (a proligo database was used) and I transfected this duplex in parallel with the siRNA Chk1 by using the same experimental conditions. As it is possible to observe in figure 5.1 Chk1 protein levels were reduced to almost undetectable levels at both 48 and 72 hours post transfection with siRNA Chk1 while the scramble siRNA and the mock untransfected samples basically showed comparable Chk1 protein levels. The β -tubulin levels were detected as internal loading control. In the same experimental conditions, to observe the mitotic phenotype, set of samples seeded on round coverslips were fixed and stained with the mitotic spindle marker α -tubulin and then with the nuclei stain DAPI as described in materials and methods. Thanks to this staining we could easily detect at the fluorescence microscope the number and the morphology of the mitotic cells present in the fixed samples both by the bright colour and very well distinguishable morphology of the mitotic spindle and by the evident chromosomes condensation of the mitotic cells. In figure 5.2 are reported two different fields corresponding to the siRNA scramble sample (left part of the figure) and the siRNA Chk1 one (right part of the figure) at 72 hours post transfection. The mitotic phenotype of the siRNA scramble sample looked normal both in the mitotic spindle and in the chromosomes morphology similarly to what observed in the mock untransfected cells (data not shown). On the contrary, as it can be easily observed in the right part of the figure 5.2, the mitotic phenotype of the siRNA Chk1 sample looked abnormal both in the mitotic spindle and in the chromosomes morphology and structure. The 48h post transfection time point showed a comparable normal mitotic phenotype both in scramble and Chk1 siRNA transfected samples (data

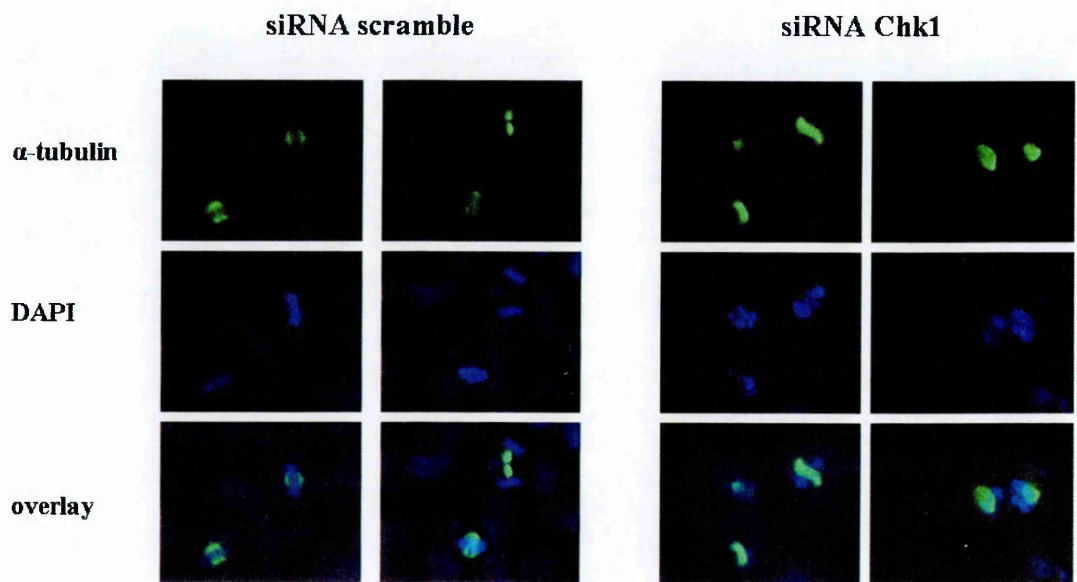


Figure 5.2 Mitotic spindle staining in HeLa cells either scramble or Chk1 siRNA transfected

Immunofluorescence analysis performed in HeLa cells, seeded on round coverslips, at 72 hours post transfection with either scramble (left part) or Chk1 (right part) siRNA. The cells on coverslips were fixed with paraformaldehyde 4% for 20 minutes and then stained with the mitotic spindle marker α -tubulin and with the nuclei stain DAPI. Two distinct fields of the siRNA scramble slide on the left and of the siRNA Chk1 slide on the right are represented including clear examples of the mitotic figures phenotype observed.

not shown). To double check that the cells presenting the abnormal mitotic spindle were in mitosis, an additional mitotic marker, the histone H3 phosphorylated at serine 10 was used to co-stain (together with α -tubulin) the scramble and Chk1 siRNA transfected samples. The costaining was possible because of the different features of the secondary antibodies used for α -tubulin and Histone 3 phosphorylated in serine 10 staining which were respectively rat-FITC conjugated and mouse Cy3 conjugated. The results observed after the costaining showed that the cells with the abnormal mitotic spindle and chromosomes morphology observed in the siRNA Chk1 transfected sample were effectively mitotic cells (figure 5.4). Figure 5.3, representing the siRNA scramble transfected cells shows that only mitotic cells (looking normal in this sample) present the double staining. This experiment was repeated several times and the abnormal mitotic phenotype observed in the siRNA Chk1 transfected cells was reproducible. Interestingly enough an accumulation of cells in mitosis after transfection with the siRNA Chk1 compared to the siRNA scramble transfected ones was observed. Three independent experiments were analysed and the percentage of mitotic cells and of the different mitotic abnormalities was calculated as depicted in figure 5.5 and as the legend of the figure further explain. The analysis revealed an increased percentage of mitotic cells in the siRNA Chk1 sample (20%).

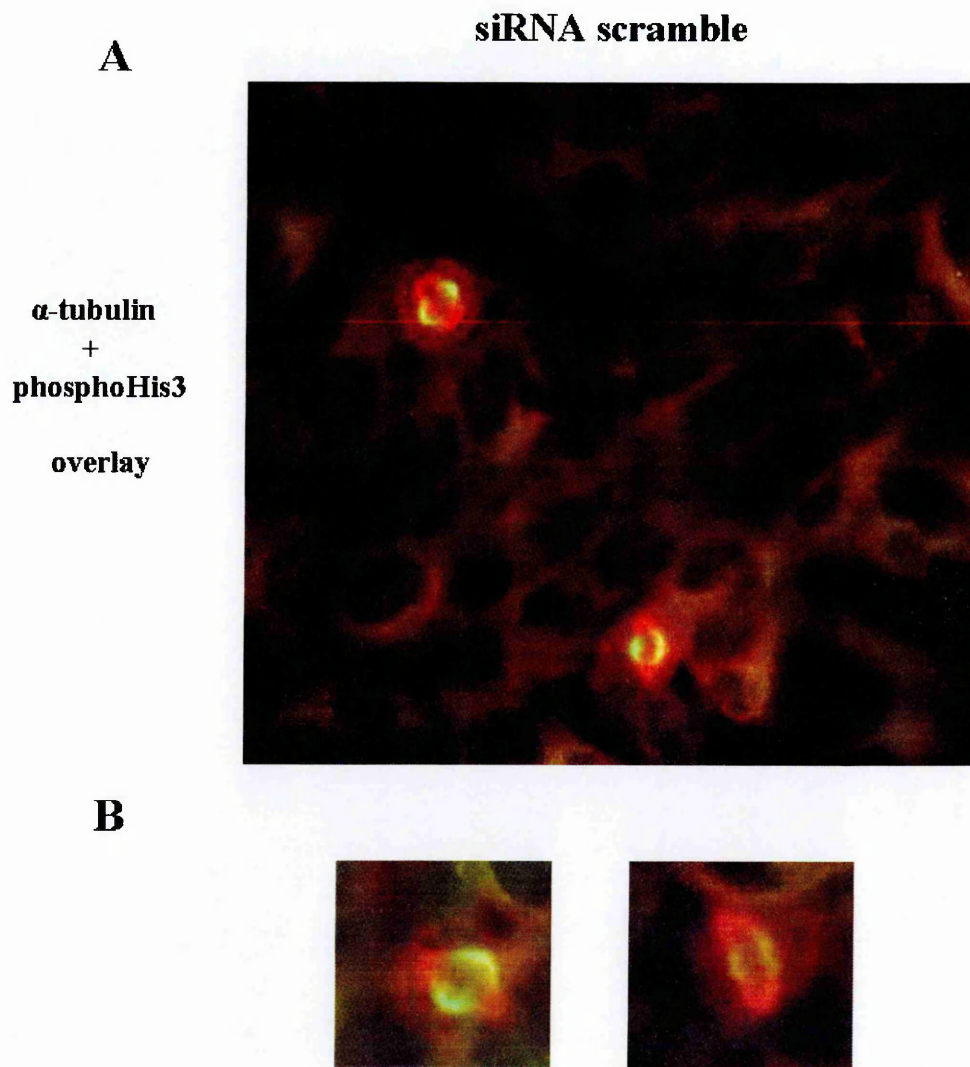


Figure 5.3 Mitotic figures in scramble siRNA transfected HeLa cells

Panel A. Immunofluorescence analysis performed in HeLa cells at 72 hours post transfection with siRNA scramble and stained with the two markers of mitosis: α -tubulin (green,Alexa Fluor 488) and Histone 3 phosphorylated in serine 10 (red,Alexa Fluor 594), like described in materials and methods. B panel represents two additional examples of mitotic cells taken from other fields of the slides.

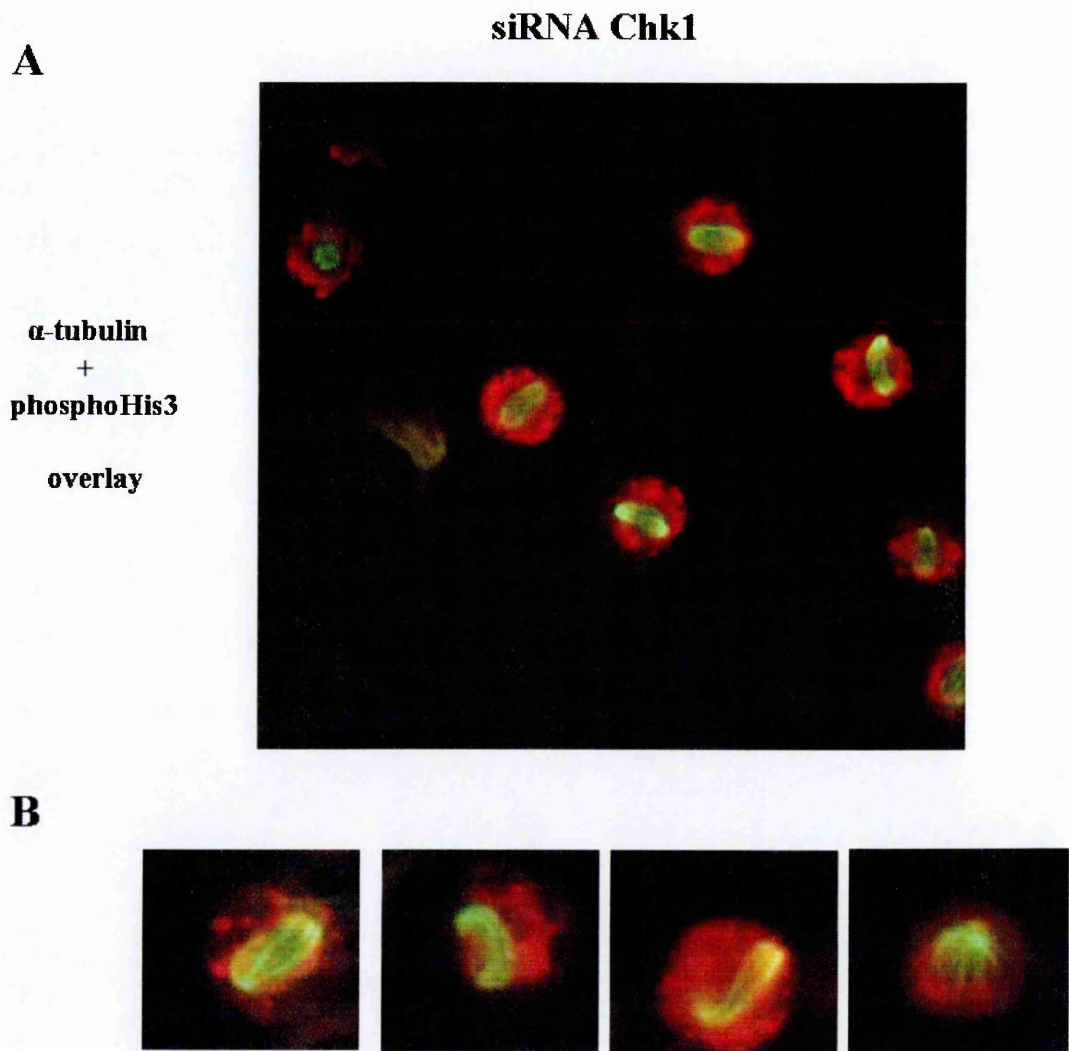
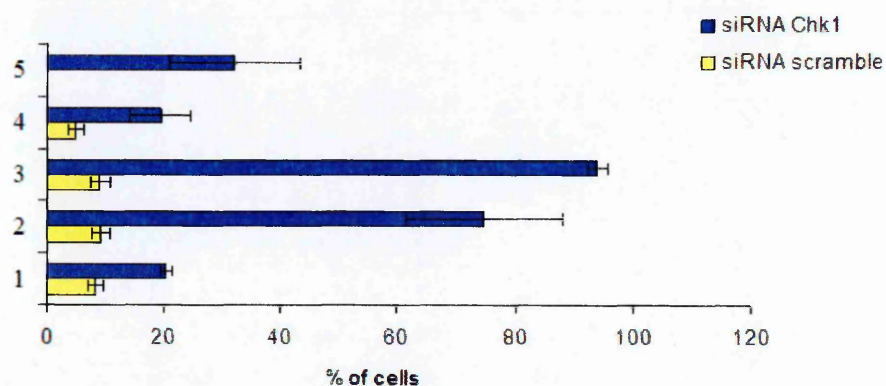


Figure 5.4 Mitotic figures in Chk1 siRNA transfected HeLa cells

Panel A. Immunofluorescence analysis performed in HeLa cells at 72 hours post transfection with siRNA Chk1 and stained with the two markers of mitosis: α -tubulin (green,Alexa Fluor 488) and Histone 3 phosphorylated in serine 10 (red,Alexa Fluor 594). B panel represents four additional examples of mitotic cells taken from other fields of the slides and confirming the abnormal phenotype observed in the Chk1 siRNA transfected sample. A “loop spindle” phenotype can be observed in the second example from the left.



| | |
|---|--|
| 1 | % of mitotic cells |
| 2 | % of mitotic cells with abnormal spindle |
| 3 | % of mitotic cells with DNA abnormalities |
| 4 | % of mitotic cells with monopolar spindle |
| 5 | % of mitotic cells presenting "loop-spindle" |

Figure 5.5 Analysis of the mitotic figures in HeLa cells transfected with either scramble or Chk1 siRNA

The data obtained are the mean \pm SD of three independent experiments. The percentage of mitotic cells was calculated among 300 cells (group 1). The percentage of the different mitotic abnormalities (groups 2-5) was calculated among 70 mitotic cells. An example of "loop spindle" phenotype is represented in figure 5.4 B (second example from the left).

5.3 MITOTIC SPINDLE ABNORMALITIES: POSSIBLE EXPLANATIONS

5.3.1 CENTROSOMES

In order to evaluate if the mitotic spindle and chromosomes abnormalities observed in the siRNA Chk1 transfected cells, could be considered as a consequence of any centrosomes positioning/separation abnormalities, we synchronized the cells at the G1/S boundary, cell cycle stage at which centrosomes duplication occur, to observe the relative positioning of the duplicated centrosomes and their separation schedule time. After 62 hours post siRNA scramble and Chk1 transfection, cells were treated with HU 2mM for 10h and then fixed at 72 h post transfection, immediately after release from HU and subsequently stained with α tubulin, DAPI and γ tubulin, a centrosomes marker. As expected, we observed a decrease in % of mitotic cells in the siRNAs scramble HU treated cells (< 5%) compared to the scramble non HU treated ones (15%) as the HU treatment did synchronize the cells at G1/S boundary thus leading to a decrease in mitotic cells (figure 5.8). In addition, as previously data in literature showed (Balczon et al., 1995), due to the HU treatment in a not functional p53 cell line (Hela/E6), the few percentage of mitotic cells observed in the siRNA scramble HU treated sample presented multipolar spindle related to multiple round of centrosomes duplication leading to centrosomes amplification. In figure 5.6 right panel, is reported an example of a multipolar spindle phenotype in a HU treated scramble siRNA transfected cells, while the left panel of this figure represents an example of a normal mitotic phenotype in the non HU treated siRNA scramble trasfected sample, with the two centrosomes well

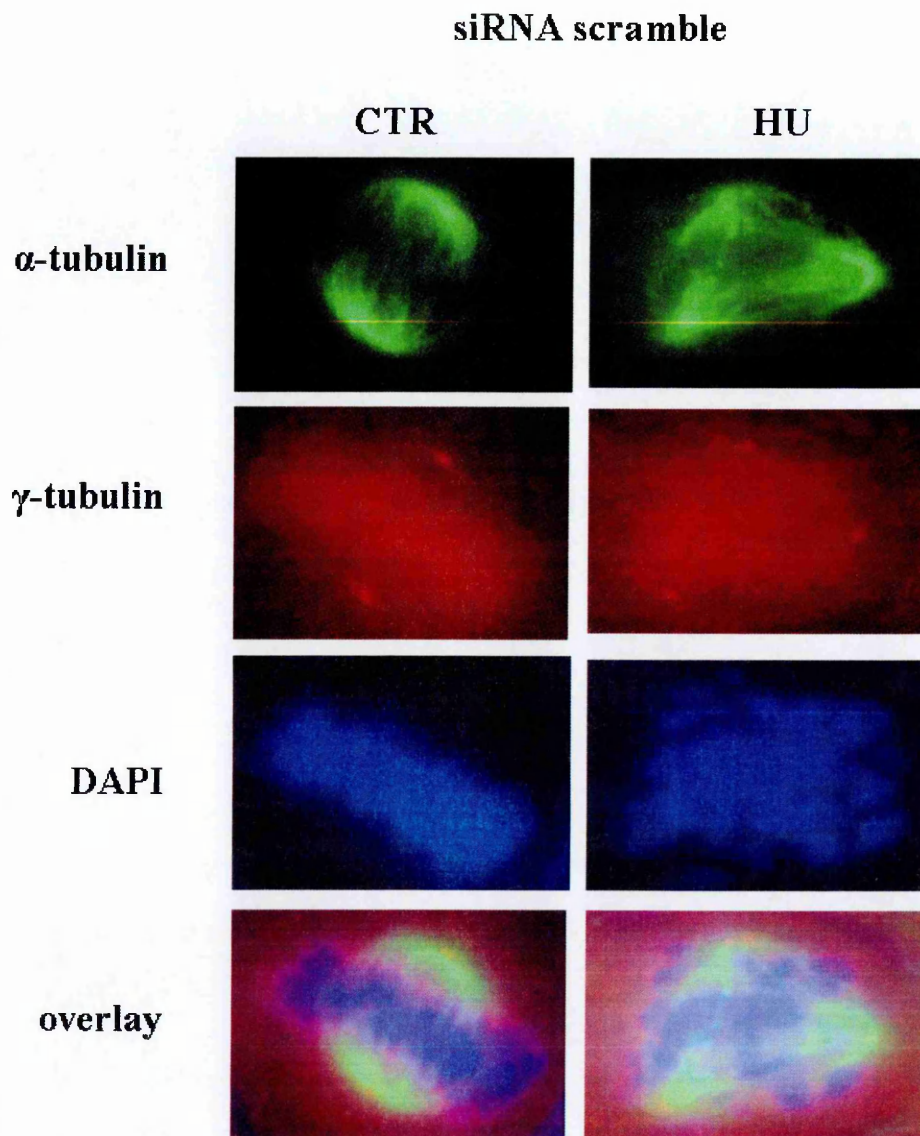


Figure 5.6 Mitotic figures in HU treated siRNA scramble transfected HeLa cells

Example of mitotic figure observed in siRNA scramble transfected HeLa cells either control (left part) or HU treated (right part). The cells were stained with the mitotic marker α tubulin, the centrosomes marker γ tubulin and DAPI.

positioned at the two spindle poles. On the other hand, in the siRNA Chk1 HU treated sample, we could not observe a decrease in % of mitotic cells (31%), compared to the siRNA Chk1 untreated sample (27%), (figure 5.8) suggesting that in this cell line the lack of Chk1 caused a defect in blocking the cells in S phase after treatment with a DNA synthesis inhibitor. We also observed centrosomes amplification in the siRNA Chk1 HU treated cells as well as in the scramble HU treated sample, although the centrosomes positioning in the siRNA Chk1 HU treated cells was altered compared to the position of the centrosomes in the multipolar mitotic figures observed in the siRNA Chk1 scramble HU treated cells. In the right panel of figure 5.7 A it is possible to observe two different examples of mitotic figures phenotype in the siRNA Chk1 HU treated samples with centrosomes not positioned and organized as in the siRNA scramble HU treated sample. In fact in one case the three centrosomes present positioned close each other in just one spindle pole, while in the other example reported in the figure, it is possible to observe the presence of two centrosomes at the level of one of the two spindle poles present. The scheme described in panel B of figure 5.7 represents the possible centrosomes orientation observed in this sample. The non HU treated siRNA Chk1 transfected samples (left panel of figure 5.7 A) basically presented the same mitotic phenotype already described in the previous paragraph. In figure 5.8 the percentage of mitotic cells and mitotic cells with multipolar spindle each group is summarized and is represented as mean \pm SD of two independent experiments. The legend of the figure further explains the criteria of this analysis.

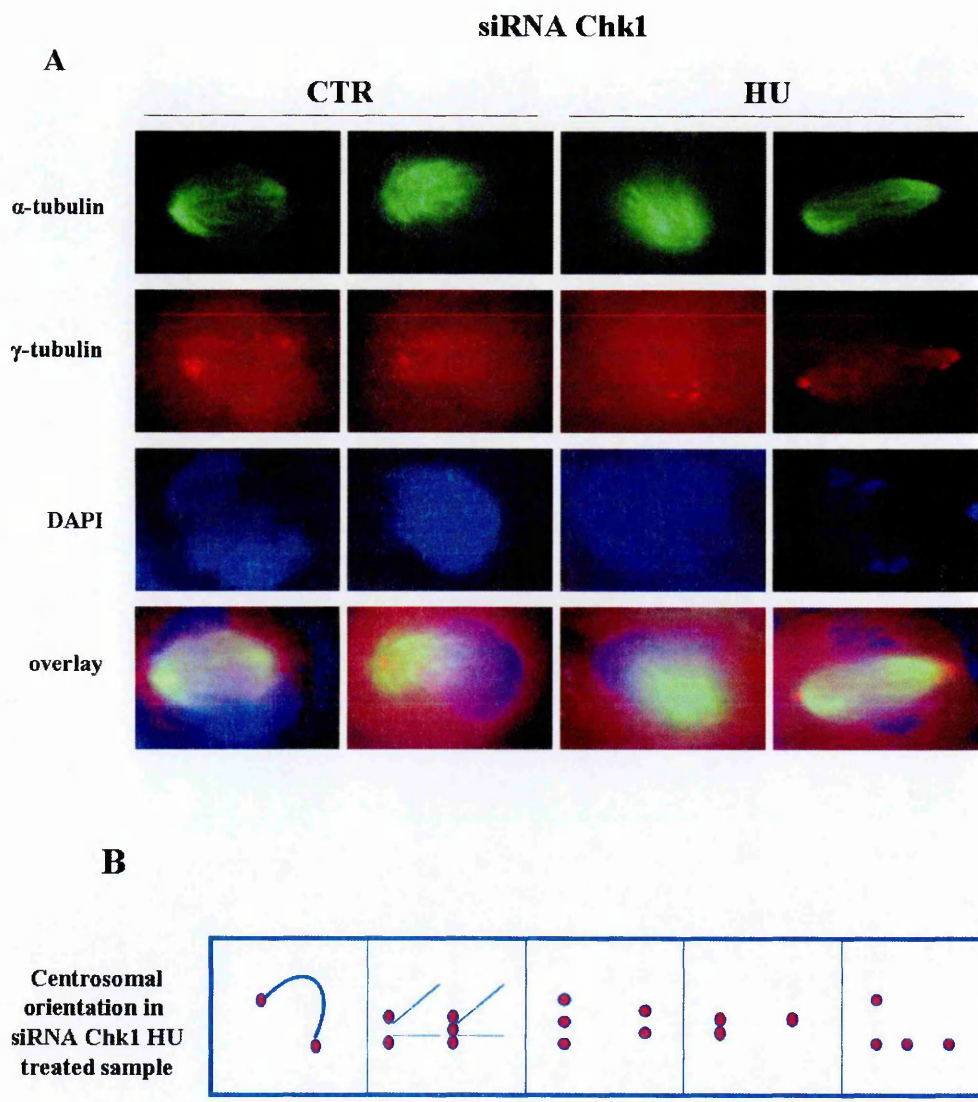


Figure 5.7 Mitotic figures in HU treated siRNA Chk1 transfected HeLa cells

Panel A. Examples of mitotic figures (two each) observed in siRNA Chk1 transfected HeLa cells either control (left part) or HU treated (right part).

Panel B. Schematic representation of the different possible centrosomes orientation observed in the siRNA Chk1 HU treated sample.

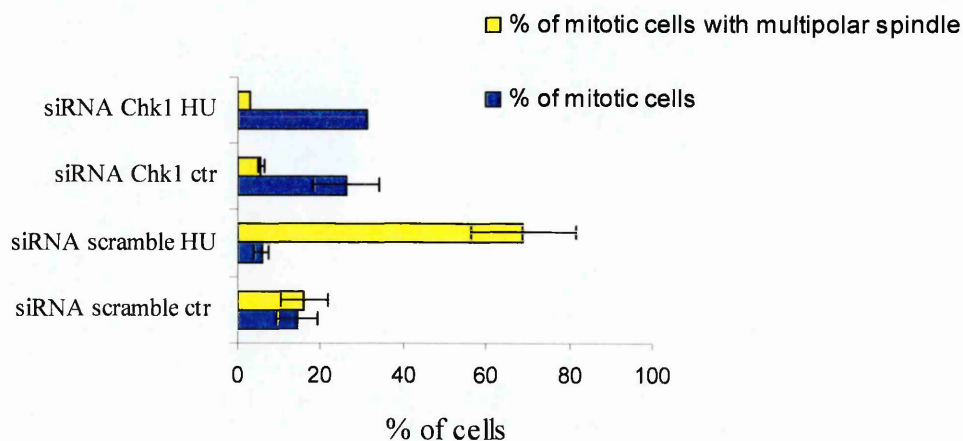


Figure 5.8 Analysis of mitotic cells in HeLa cells transfected with either scramble or Chk1 siRNA and either treated or not with HU

Schematic representation of the percentage of mitotic cells and mitotic cells with multipolar spindle in the 4 samples studied. The % of mitotic cells each group was calculated among 300 cells, while the % of mitotic cells with multipolar spindle was calculated among 40 mitotic cells each group. The data represent the mean \pm SD of two independent experiments.

5.3.2 PREMATURE MITOSIS

To further investigate the nature of the accumulation of abnormal mitotic figures in siRNA Chk1 transfected cells, a FACS analysis was performed to study the cell cycle profile of siRNA Chk1 transfected cells compared to the siRNA scramble ones at 72 hours post transfection (time point at which we could detect by IF the mitotic figures abnormalities). An increased percentage of S population in siRNA Chk1 transfected cells *versus* siRNA scramble ones was observed (figure 5.9 A upper panel). An additional mitotic marker, cyclin B1, whose expression starts in late S phase and persists up to the metaphase-anaphase transition, was picked up to study its cell cycle dependent expression by FACS analysis. This analysis revealed an accumulation of cyclin B1 positive population in a high percentage in S phase suggesting that probably part of the S population represents the “mitotic population” observed by the microscope analysis (figure 5.9 A lower panel). In figure 5.9 C the bi-parametric representation of cyclinB1-FITC and DNA content (PI) is depicted, clearly showing a higher amount of cyclin B1 positive cells in the S phase of the siRNA Chk1 sample compared to the siRNA scramble sample. In figure 5.9 B the Western blot analysis representing the control of the working downregulation of the Chk1 protein, confirmed the increased levels of cyclinB1 in the siRNA Chk1 transfected sample. We hypothesized that in HeLa cells the lack of Chk1 would lead to a premature mitosis before the completion of DNA replication. Probably the increased % of cells in mitosis observed by IF could include part of the S phase population observed by FACS, thus having a <4N DNA content. This would explain the incomplete chromosomes condensation observed in these abnormal mitotic figures.

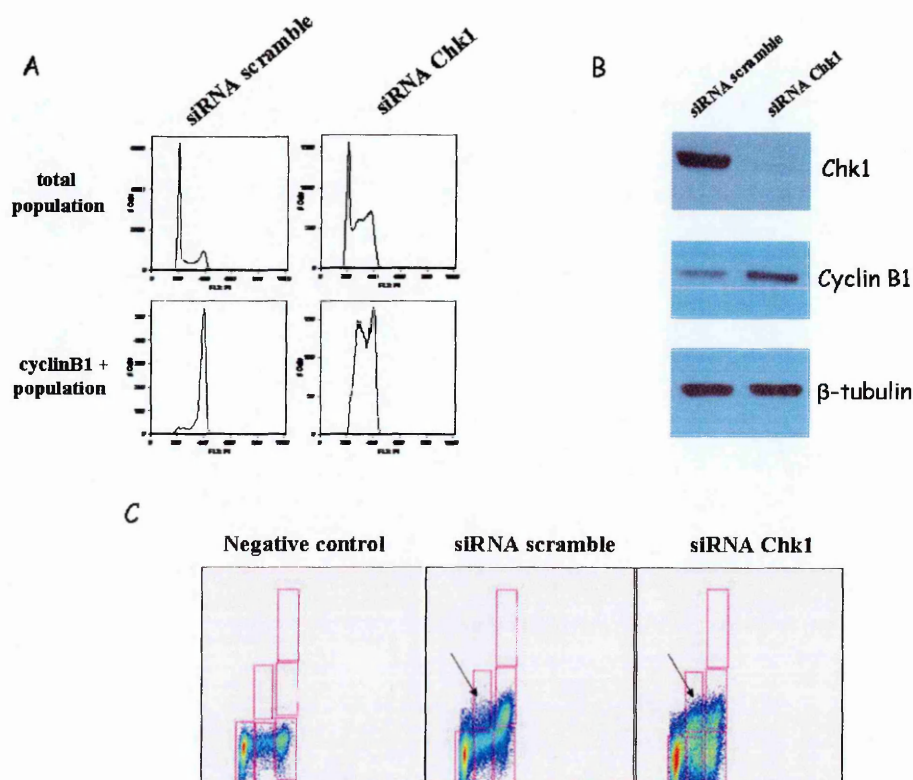


Figure 5.9 Analysis of the cyclin B1 levels in HeLa cells at 72 hours post transfection with either scramble or Chk1 siRNA

Panel A. Ethanol 70% fixed cells stained with cyclin B1 and propidium iodide. FACS analysis was performed. The total population (upper panel) and the cyclinB1 positive population (lower panel) cell cycle profile of the two samples studied are represented.

Panel B. Chk1, cyclinB1 and β tubulin protein levels detected by Western blot analysis in the scramble and Chk1 transfected samples at the same time point (72h) at which FACS and IF analysis were performed.

Panel C. Bi-parametric representation of cyclinB1 and DNA content in the control negative sample (secondary antibody only), siRNA scramble and siRNA Chk1 samples. The arrows specifically indicate the cyclin B1 population in S phase.

5.4 INVESTIGATION ON THE POSSIBLE PHYSIOLOGICAL ROLE OF CHK1 IN ADDITIONAL SOMATIC CELL LINES

In order to investigate if the accumulation in S phase observed by FACS analysis in the Chk1 siRNA transfected HeLa cells, associated with a possible premature mitosis before completion of DNA replication, could be extended also to other cellular lines we studied the cell cycle perturbation in parallel in different cell lines transfected with either scramble or Chk1 siRNA. Standard transfections conditions were used to transfect a wider spectrum of human somatic cell lines. We repeated this experiment in the HCT-116 p53 $+/+$ and p53 $-/-$ cell line, although previous data did not show any alteration in cell cycle profile at 72h post Chk1 downregulation, (figures 4.8 and 4.9) in order to double check if the introduction of a mismatch in the double strand siRNA sequence would possibly modify the features previously observed. We also studied at 72 hours post transfection the cell cycle profile of two ovarian cancer cell lines, SKOV-3 and A2780 and of the osteosarcoma cell line U2OS. As it is depicted in figure 5.10 in the HCT116 p53 $+/+$ and p53 $-/-$ a comparable cell cycle profile in the cells with and without the expression of Chk1 was observed (confirming the data previously shown). In the SKOV-3 ovarian cell line even if it was possible to observe a slight accumulation of cells in S phase in the siRNA Chk1 transfected sample, this was not accompanied by any abnormality in the phenotype observed by IF (data not shown). On the other hand in the A2780 cell line a comparable cell cycle profile in the absence of Chk1 as comparing with that one observed in the scramble siRNA transfected sample was observed. The immunofluorescence analysis conducted in parallel on these cell lines, revealed no mitotic phenotype abnormalities in the absence of Chk1 (data not shown). On the

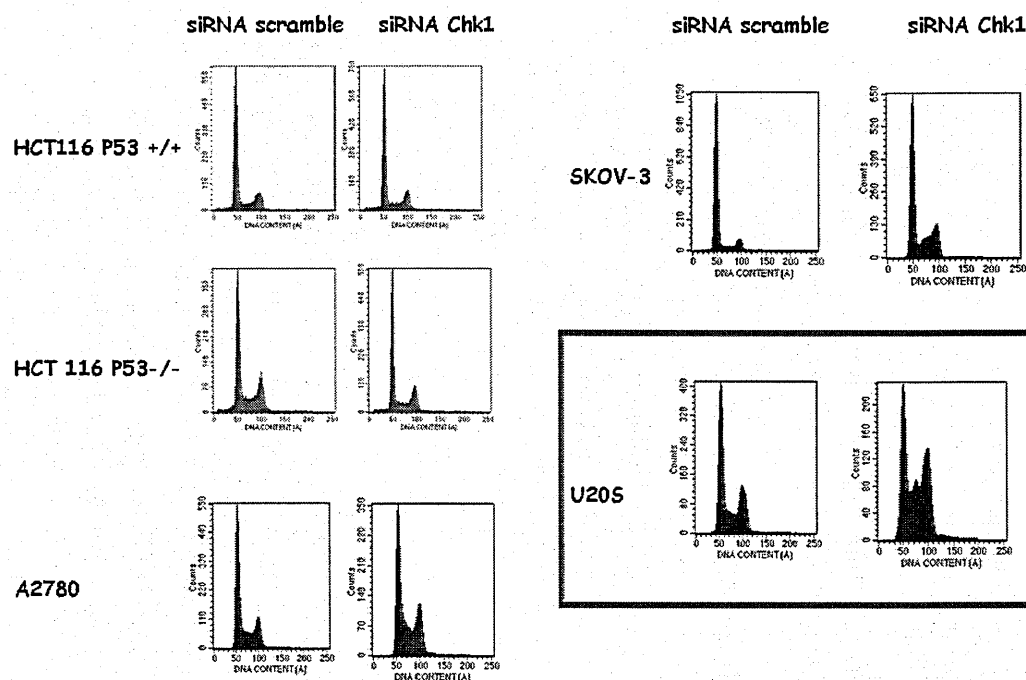


Figure 5.10 Cell cycle profiles of siRNA Chk1 transfected cells

FACS analysis performed on different human somatic cancer cell lines at 72 hours post transfection with either scramble or Chk1 siRNA.

contrary, by analysing the cell cycle profile of the U2OS cell line it was possible to observe an accumulation of cells in S and G2-M phase, similarly to what was observed in the HeLa cell line (figure 5.9).

The U2OS cell line was then further studied. Figure 5.11 A shows the Chk1 and actin protein levels detected by Western blot analysis to confirm the downregulation of the protein of interest at 72 hours post transfection. To investigate the cell cycle specific cyclin B1 expression levels, the cells were double stained with cyclinB1 and with ToPro and the FACS analysis was performed at 72 hour time point. As it is possible to observe in figure 5.11 B, the siRNA Chk1 transfected cells apparently presented a consistent accumulation of cells in G2-M phase although, the percentage of positive cyclinB1 cells in G2-M was comparable in the siRNA scramble and Chk1 samples. The consistent amount of cyclin B1 negative cells in G2-M in the siRNA Chk1 transfected sample (represented by R3 box) led to the hypothesis that probably part of the apparent G2-M population observed could represent the G1 ratio of the tetraploid population (S and G2-M of this population represented by box R9) thus explaining the lack of expression of cyclin B1. No significant cyclinB1 expression was observed in the S phase of the Chk1 siRNA transfected sample suggesting that probably the S and G-M accumulation observed in U2OS cells has a different biological significance respect to the one hypothesized in HeLa cells. The mitotic figures were also studied in this cell line by staining with α tubulin and DAPI thus confirming this hypothesis. In figure 5.12 the immunofluorescence analysis at 72h post siRNA scramble (upper part) and siRNA Chk1 (lower part) transfection is depicted. No increase in the % of cells with mitotic spindle could be observed in the siRNA Chk1 transfected cells compared to the siRNA scramble

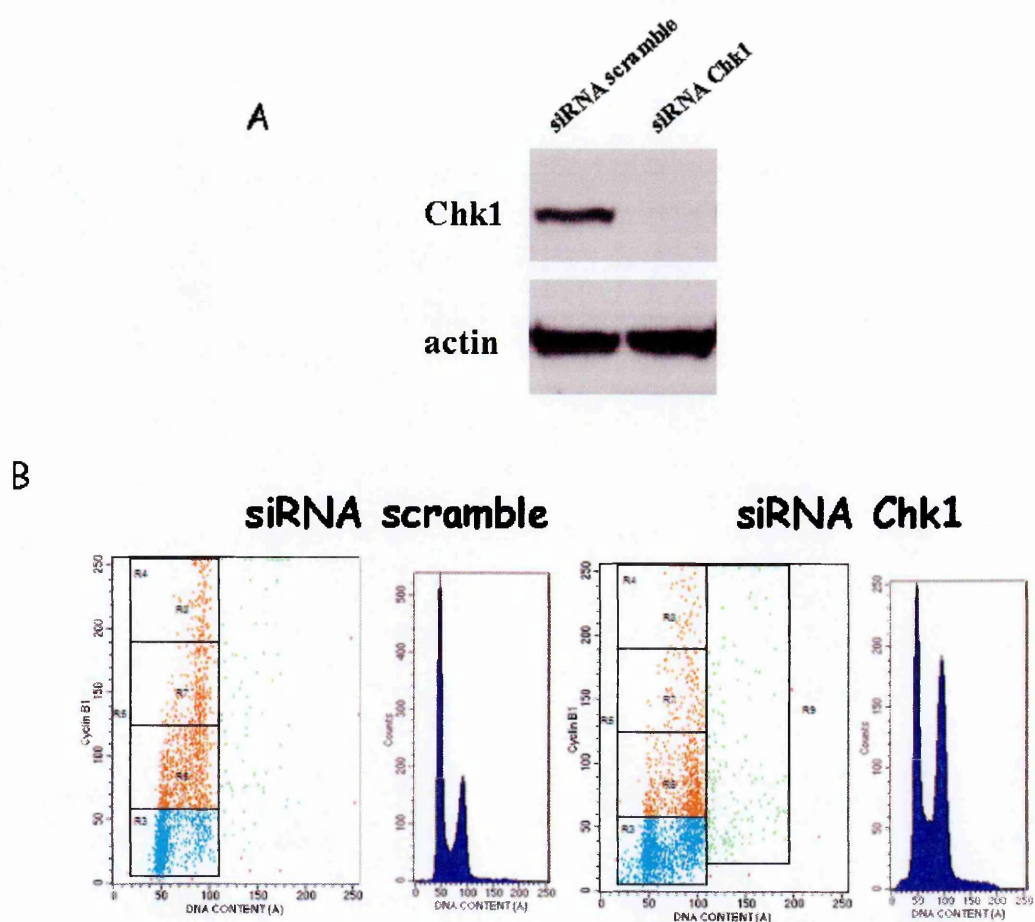


Figure 5.11 Analysis of the cyclin B1 levels in U2OS cells at 72 hours post transfection with either scramble or Chk1 siRNA

Panel A. Chk1 and actin protein levels detected by Western blot analysis performed at 72 hours post transfection with either scramble or Chk1 siRNA.

Panel B. Cell cycle specific expression of cyclin B1 performed on siRNA scramble and Chk1 transfected U2OS cells. The biparametric analysis and the simple DNA content are represented for each sample. The R3 box represents the cyclin B1 negative population while R6, R7 and R8 represent different levels of expression of cyclin B1. R9 box in the siRNA Chk1 sample represents the tetraploid population present in this sample.

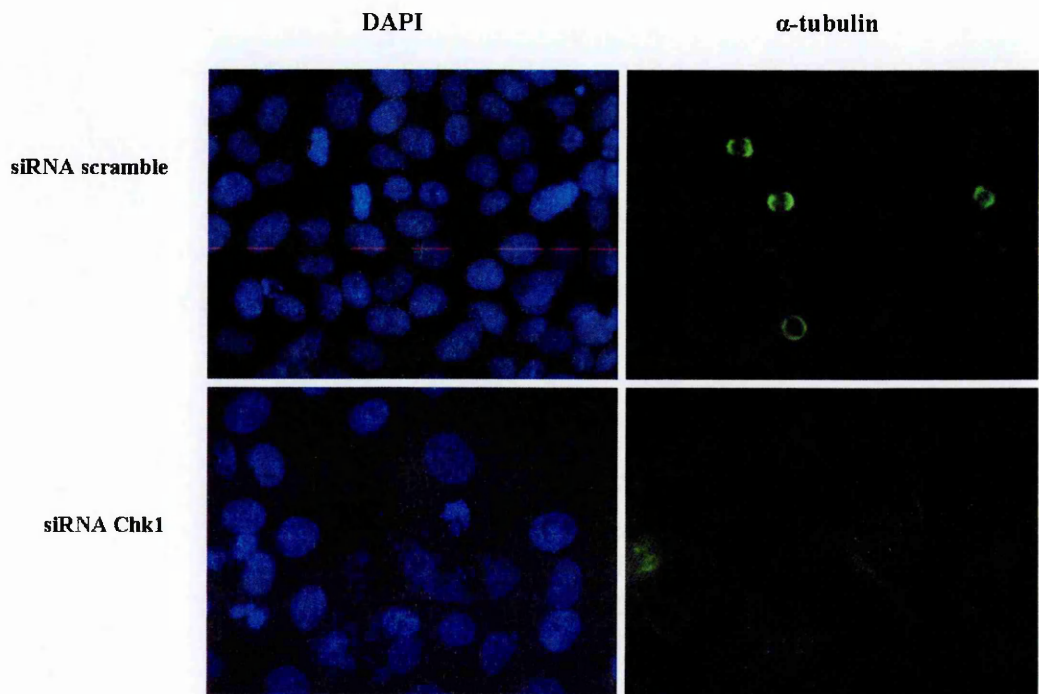


Figure 5.12 Mitotic spindle staining in U2OS cells either scramble or Chk1 siRNA transfected

Immunofluorescence analysis after staining with α tubulin and DAPI in U2OS cells either lacking or not Chk1 expression.

transfected ones. Only few of the mitotic cells in the siRNA Chk1 sample appeared abnormal, but it was easily possible to detect an increased percentage of cells undergoing death and a higher incidence of cells with micro-polynuclei. On the other hand, the siRNA scramble transfected cells looked normal.

The cell growth of the U2OS cells transfected either with scramble or Chk1 siRNA was studied and it was possible to see that, starting from 72h post transfection, cells lacking Chk1 expression stopped growing and/or started dying. (figure 5.13). This is a different result if compared with the cell growth curve obtained from the HCT116 cell lines where we could never detect cell growth differences in the absence of Chk1 (see figure 4.7 for example). In fact in figure 5.14, to further underline this difference, the relative % of live cells considering the scramble siRNA sample as 100%, is reported in the HCT116 p53+/+ and p53-/- and in the U2OS cell line at 96 hours post transfection with either scramble or Chk1 siRNA. As it is possible to observe in the figure, only U2OS cells survival was compromised by the lack of Chk1 expression.

To understand if apoptosis could be a possible mechanism responsible for the death of the cells lacking Chk1 expression, the sulforodamine/DAPI staining was performed and confirmed the presence of apoptotic morphology in the siRNA Chk1 transfected cells while the siRNA scramble transfected cellular morphology looked normal (figure 5.15). In addition in figure 5.16 the decreased levels of procaspase-3 observed by Western blot analysis would further suggest that death by apoptosis is occurring. In figure 5.17, lamin B staining confirmed the presence of apoptotic cells which have lost nuclear envelope integrity, while the micro-polynuclei still have intact lamin B. The significant increase in cell volume and the formation of micronuclei observed in the siRNA Chk1 transfected

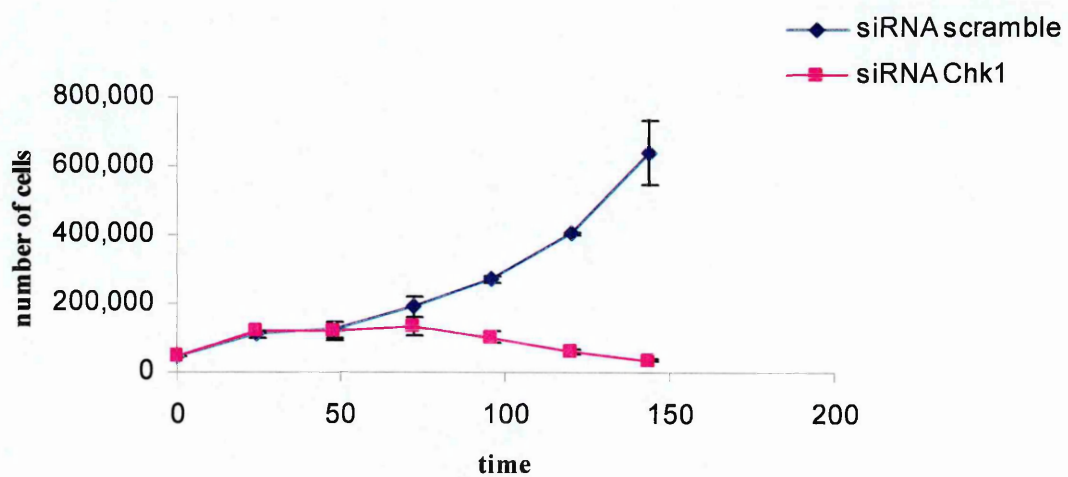


Figure 5.13 Cell growth curve of U2OS cells either scramble or Chk1 siRNA transfected

The cells were counted at 24, 48, 72, 96, 120 and 144 hours post siRNAs transfection.

The data represent the mean \pm SD of two independent experiments.

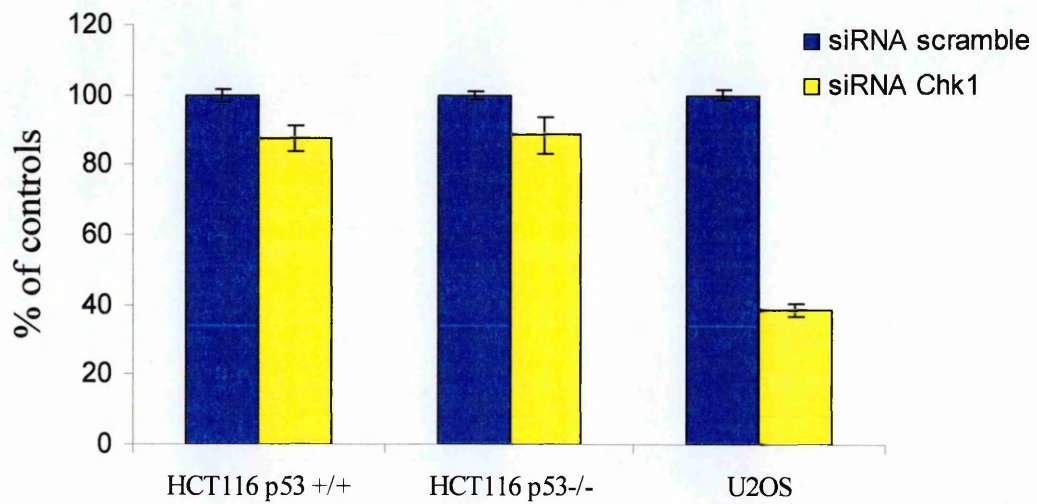


Figure 5.14 Cell survival at 96 hours post siRNA Chk1 transfection in different cell lines

Schematic representation of the percentage of cells still surviving at 96 hours post transfection with siRNA Chk1 in HCT-116 p53 +/+, HCT-116 p53 -/- and U2OS. Data are expressed as relative % of scramble treated cells and are the mean \pm SD of two independent experiments.

U2OS cells (orange arrows in fig. 5.15 and fig. 5.17) would let think about another additional death mechanism which is still under investigation.

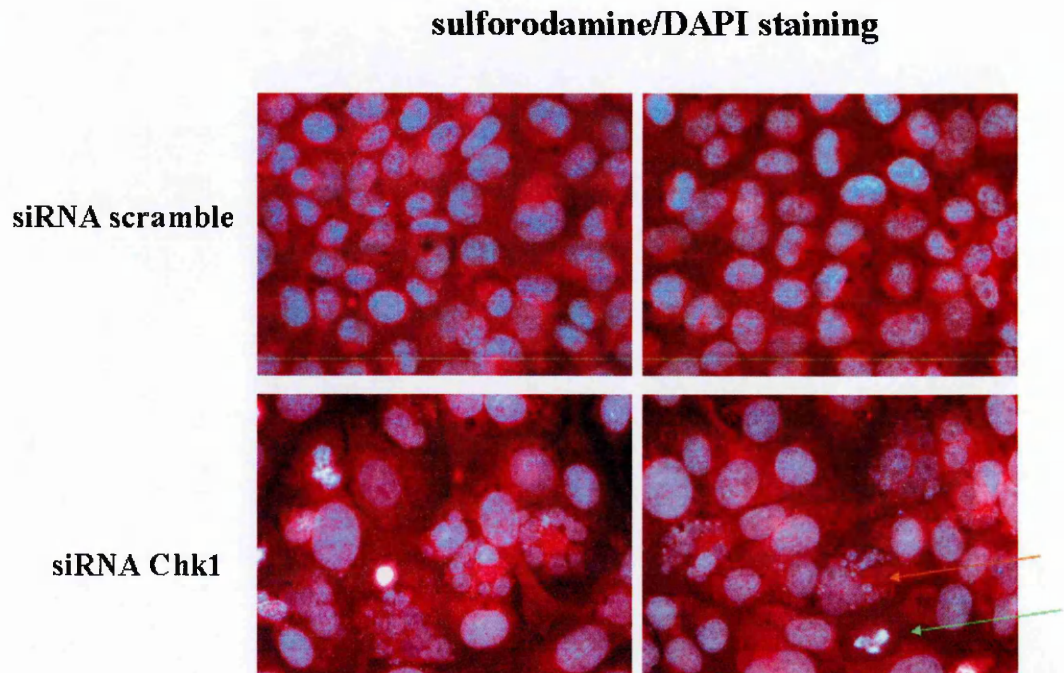
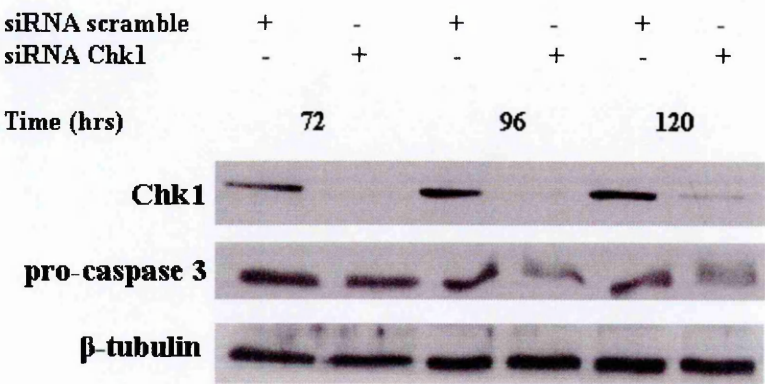


Figure 5.15 Sulforodamine/DAPI staining of U2OS cells either scramble or Chk1 siRNA transfected

The green arrow outlines a typical apoptotic morphology while the orange one evidences an example of cell presenting micro-polynuclei which appears very frequent in this cell line lacking Chk1 expression. Two distinct fields of the siRNA scramble slide (upper part) and of the siRNA Chk1 slide (lower part) are represented.

A



B

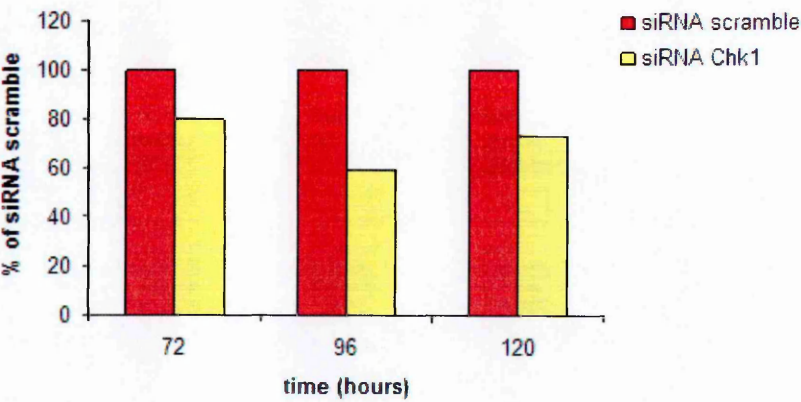


Figure 5.16 Pro-caspase 3 levels to detect apoptosis in siRNA Chk1 U2OS transfected cells

Panel A. Chk1, pro-caspase 3 and β-tubulin protein levels detected by Western blot analysis.

Panel B. Densitometric analysis of the levels of pro-caspase 3. Data are expressed as percentage of siRNA scramble relative controls and represent the ratio between pro-caspase 3 and β-tubulin.

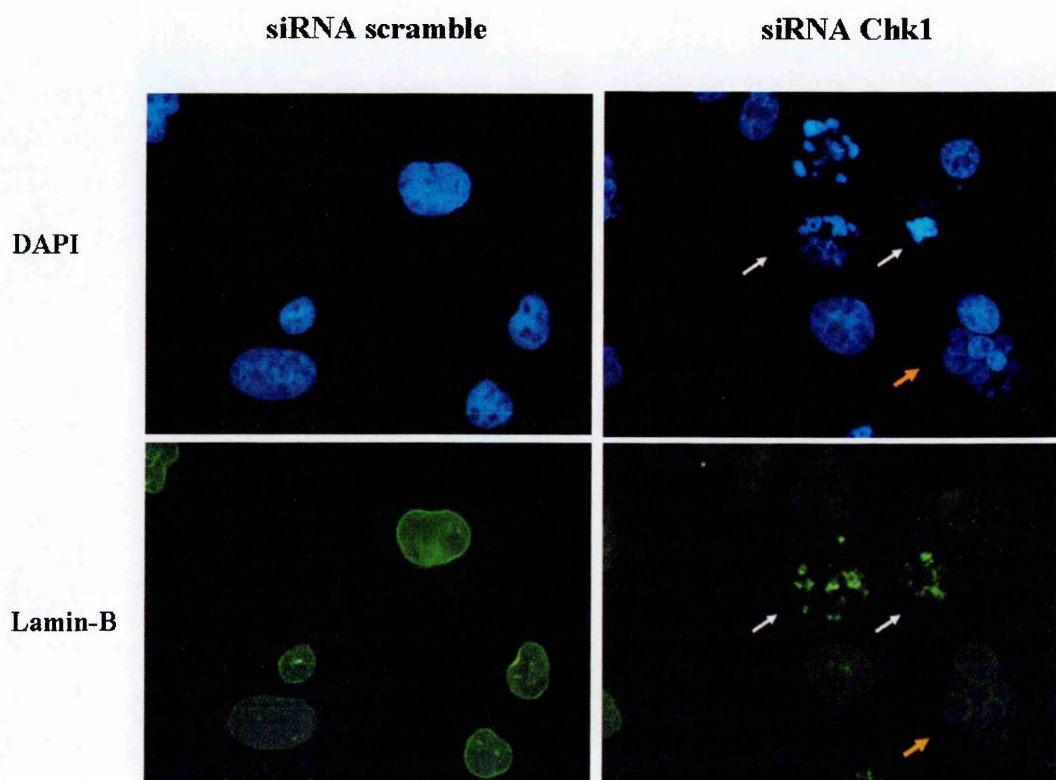


Figure 5.17 Lamin B staining in U2OS cells either scramble or Chk1 siRNA transfected

Immunofluorescence analysis conducted on siRNA scramble and Chk1 transfected cells fixed at 72 hours post transfection and stained with lamin B and DAPI. Nuclear envelope features in apoptotic cells (white arrows) and in cells with micro-polynuclei (orange arrow) are depicted.

5.5 DISCUSSION

During the time I spent in the laboratory directed by Yolanda Sanchez at Cincinnati University, US, my work, subject of this chapter, mainly focused on the investigation of the physiological role of the protein kinase Chk1 in human somatic cancer cell lines. Its role, in fact, appears still controversial although in the last years several groups gave some insights such as for example Gillespie and his group with the DT40 avian lymphoma cell line Chk1^{-/-} characterization and Lam et al. with the Chk1 heterozygous and homozygous KO conditional breast tissues characterization. The starting point of my investigation was to further understand the role of the protein kinase Chk1 in centrosome biology as recent experimental evidences attributed to Chk1 a role in the biology of these small component of a cell (Kramer et al., 2004 and data unpublished in sanchez lab). Again the siRNA technique was used to downregulate the Chk1 kinase protein and to investigate in HeLa cells, on a possible role played by this protein in determining a correct mitotic phenotype. Centrosomes in fact are involved in the mitotic spindle formation during mitosis, important for segregation of chromosomes and for a faithful transmission of the entire genome to the daughter cells. The data obtained in this cell line revealed that the lack of Chk1 would lead to a defective mitotic spindle assembly accompanied by abnormal chromosomal condensation as the data depicted in figures 5.2-5.5 underlined. To understand the biological significance of the abnormal mitotic phenotype observed in cells lacking Chk1 expression, different hypotheses were made and related experiments were performed. In the first instance I tried to explain the abnormal mitotic phenotype with a possible (still not clear) role played by Chk1 in

determining centrosomes positioning/orientation as the data described in the paragraph 5.2 would suggest. In the second instance the cell cycle profile by FACS analysis was studied to deeply investigate the nature of the increased % of mitotic cells in the siRNA Chk1 transfected cells observed by IF. The results revealed that the accumulation of cells in S phase was correlated with a high cyclin B1 expression in this S phase population in the siRNA Chk1 transfected sample. These data suggest that in HeLa cells the absence of Chk1 induces cells to undergo mitosis before the completion of DNA replication. This observation is in line to what has been recently observed (Niida et al., 2005) in the conditional Chk1 deficient mouse ES cells, where the authors detected by microscopic laser scanning cytometry, Histone 3 phosphorylated in serine 10 positive cells with a DNA content of less than 4N. These data also corroborated with the observation made by Lam et al. by studying the Chk1 heterozygous and homozygous breast tissues where they could observe nuclei positive for both BrdU and for Histone 3 phosphorylated in serine 10 staining, respectively replication and mitotic markers (Lam et al., 2004). The abnormal mitotic phenotype observed in siRNA Chk1 transfected HeLa cells appeared similar to that one observed by Gillespie and his group in lymphoma DT40 Chk1 $-/-$ cell line after aphidicolin treatment while they did not observe this phenotype in the non treated Chk1 $-/-$ cells (fig. 5.18) (Zachos et al., 2005). In lymphoma DT40 Chk1 $-/-$ aphidicolin treated cells the entry in mitosis from S phase has been explained with the loss of replication capacity determined by the degeneration of viable replication structures which are normally stabilized by Chk1 during replication arrest. The premature mitosis before completion of DNA synthesis observed in HeLa cells in the absence of replication arrest could be

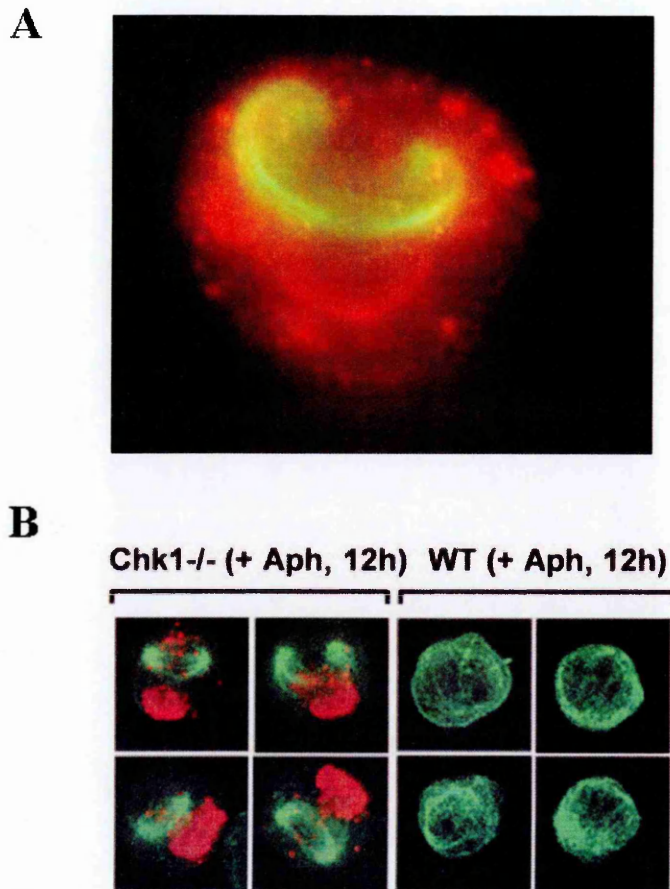


Figure 5.18 Possible explanation for the abnormal mitotic phenotype observed in siRNA Chk1 transfected HeLa cells

Comparison between the “loop spindle” morphology observed in siRNA Chk1 transfected HeLa cells and the mitotic phenotype observed in avian lymphoma DT40 Chk1^{-/-} cell line isolated by Zachos et al. and treated with Aphidicolin for 12 hours.

Panel A. Higher magnification of a “loop spindle” mitotic cell observed in HeLa cells at 72 hours post transfection with siRNA Chk1 and co-stained with anti α -tubulin and anti Histone 3 phosphorylated in serine 10.

Panel B. Figure derived from a paper by Zachos et al. where they showed the mitotic phenotype observed in Chk1^{-/-} cells after replication block induced by aphidicolin. (Panel B modified by Zachos et al., 2005).

explained by the possibility that in this cells line Chk1 is also necessary for the maintenance of viable replication structures during normal S phase progression. This hypothesis had been made also to explain the formation of DNA breaks and pH2AX activation recently observed by Bartek and his group in U2OS cells where Chk1 has been inhibited either by siRNA or by specific inhibitors (Syljuasen et al., 2005). The hypothesis of the entry in mitosis before the completion of DNA synthesis could in part also explain the abnormal centrosome positioning/orientation observed and described in the paragraph 5.2.1 as probably in these cells together with the incomplete chromosomal condensation, centrosomes separation (which usually happens in late G2) did not completely occur as well.

To investigate whether this phenomenon was restricted to the HeLa cell line, siRNA Chk1 transfection was conducted on a wider spectrum of somatic cell lines. Among 5 different somatic cell lines only the U2OS osteosarcoma cell line presented a similar S phase accumulation although the IF analysis revealed an increased percentage of cells undergoing death and no accumulation of cells with mitotic spindle. The cell growth curve showed a substantial arrest and/or death of these cells in the absence of Chk1 which we could never observe in the previously studied colon carcinoma cell system HCT116 where this protein kinase is unlikely to be essential for the viability of the cells. These data suggest that the lack of Chk1 may have different consequences depending on the intrinsic genetic background of the cell line studied.

Preliminary data, such as the DAPI/sulforodamine staining and the investigation of the levels of pro-caspase 3, revealed that apoptosis is occurring in U2OS cells when transfected with the siRNA Chk1. A high percentage of cells presenting micro-

polynuclei was also observed, suggesting that these cells could be dying by another different mechanism. Alternatively the cells presenting this morphology could represent the step just prior to apoptosis. The cell cycle specific expression of cyclin B1 revealed that a large part of the diploid G2-M cells did not express the mitotic marker thus hypothesizing that a certain percentage of G1 cells belonging to the new tetraploid population could also be included in this population. Recently, another group had studied the U2OS cells lacking Chk1 both by siRNA transfection and by specific inhibitors finding out that in this cell line the lack of Chk1 causes a series of consequences such as increased DNA replication, phosphorylation of ATR targets and DNA breakage, although they could never detect sign of apoptotic nuclear morphology, differently from the results just described.

CHAPTER 6

GENERAL DISCUSSION

The experiments described in this thesis were aimed at elucidating the molecular mechanisms regulating the G2 checkpoint after DNA damage and at investigating the key protein kinase regulator of this checkpoint, Chk1, as a potential therapeutic target for new cancer treatments. The main idea of this strategy is to combine standard chemo or radiotherapy with drugs that inhibit Chk1 in order to inhibit the S and G2 checkpoints both activated by this kinase protein in response to DNA damage, with the aim to increase the therapeutic index. Cancer cells, could be more sensitive to such combined treatments with respect to normal cells, since they lack the normal G1 checkpoint, due to the high incidence of p53 mutations in human tumors, and rely only on the S and G2 checkpoints to repair the damage (Dixon and Norbury, 2002). Some G2 abrogators are currently proposed to test this hypothesis, and promising results have been obtained with some of these inhibitors such as UCN-01 or CEP-3891 (Syljuasen et al., 2004). The mechanisms on which the observed increased cytotoxic activity is based, are still to be defined. In fact, these inhibitors appear to be far from highly specific for Chk1 thus masking the effective contribution of this kinase on the effects observed (Bunch and Eastman 1996; Husain et al. 1997; Monks et al. 2000). A large part of the work described here was aimed at searching for a suitable strategy to “specifically” inhibit the function of Chk1 in human somatic cancer cell lines growing *in vitro* with the final goal to increase the cytotoxic activity of anticancer agents in cells with either an intact or a compromised G1 checkpoint. For this purpose I focused the investigation on the cleanest isogenic cellular system available in our laboratory, represented by the colon carcinoma cell line HCT-116 wt and its isogenic systems with either p53 or p21 inactivated by homologous

recombination (thus having a compromised G1 checkpoint). In chapter 4 the different strategies aimed at inhibiting Chk1 are described (chapters 4.1-4.4). The most successful strategy, which made this investigation possible was the highly specific and efficient small interfering RNA technique. The Chk1 siRNA chosen after accurate validation permitted the reduction of Chk1 expression to almost undetectable levels in the isogenic cellular system studied (chapter 4.5). The data obtained (sections 4.5.1-4.5.2) indicate that on a compromised G1 checkpoint background it is possible to achieve higher abrogation of the G2 block associated with an increased sensitivity to treatment with DNA damaging agents than on an intact G1 checkpoint background. These experiments could also be validated in an “*in vivo*” setting and the siRNA strategy might render this objective possible. In preparation of such experiments in mice the specific Chk1 cDNA target has been cloned in a specific tetracycline inducible plasmid under the control of an H1 promoter and cellular clones stably expressing the Chk1 siRNA have already been obtained (chapters 4.6.1-4.6.2). Once the functionality of this siRNA inducible expressing system will have been confirmed in mice, the next crucial step will focus on the investigation of the chemosensitivity of these tumors, where it is possible to downregulate Chk1 upon addition of tetracycline in mice drinking water. By using the small interfering RNA strategy, the protein kinase Chk2 was downregulated in parallel in the same experimental isogenic system alone or together with the protein kinase Chk1 (sections 4.5.1-4.5.2). The results obtained support the notion that Chk2 does not have any key role in mediating the cellular response to DNA damaging agents, at least in the systems studied, although overlapping functions with the protein kinase Chk1 have been attributed to Chk2 (Bartek and Lukas, 2003). Taken together these data further

corroborate Chk1 as possible specific target for inhibition to lead to novel cancer therapy. The results reported in chapter 3 are aimed at increasing the knowledge of the links between the putative therapeutic target Chk1 and the different proteins participating in the checkpoint response. In particular to define the role of the oncosuppressor p53 in the regulation of Chk1 better, the structural and functional characterization of the 5' flanking region of the human Chk1 gene was investigated. Previous studies had shown a reciprocal regulation between Chk1 and p53. Chk1 can contribute to the phosphorylation and activation of p53 soon after DNA damage, and at later time points Chk1 would be downregulated in a p53 dependent manner with the aim to resume the cell cycle once the damage has been repaired (figure 3.1) (Damia et al., 2001; Gottifredi et al., 2001). The understanding of the inter-relationships existing among the different checkpoint pathways (G1, S, G2-M checkpoints) is of particular importance in determining the final cellular response to anticancer treatments. The results (chapter 3.5.1) suggest that the oncosuppressor p53 is unlikely to need to bind directly to the DNA for negative regulation of the Chk1 gene, as the mutated form of p53 in the DNA binding domain was equally effective in downregulating the Chk1 promoter. The results described in chapter 3.5.3 also show that E2F1 can positively regulate the Chk1 promoter. This interesting connection can be placed in a larger scenario, in which both Chk1 and E2F1 regulate the cell cycle progression under unstressed conditions. Recent experimental evidence suggests that Chk1 has a crucial role in regulating the Cdc25A turnover in the absence of DNA damage by direct phosphorylation on multiple Cdc25A residues (Sorensen et al., 2003; Zhao et al., 2002; Sorensen et al., 2004). By regulating Cdc25A turnover, Chk1 would modulate the

Cdc25A phosphatase activity, and thereby its capability to activate cyclin E/A cdk2 complexes that regulate S phase entry and progression, respectively. Interestingly Cdc25A expression can be transcriptionally regulated by E2F1. The complex role played by E2F1 in regulating the promotion of the S phase, would include the finely coordinated induction of a powerful inducer of DNA replication like Cdc25A, and of the inhibitor of this activity, such as Chk1, which by regulating the Cdc25A turnover would prevent the unscheduled and excessive initiation of DNA replication. The p53 dependent inactivation of the E2F1 transcriptional factor, which occurs after DNA damage, through the transactivation of p21, can indirectly explain the p53 dependent downregulation of the Chk1 gene.

The data obtained also suggest the existence of a positive regulation of the Chk1 promoter by the ΔN isoform of p73 α and a dominant negative action on the p53 activity (chapter 3.5.2), thus suggesting that upon damage a $\Delta Np73$ dependent upregulation of Chk1 protein levels would help in inducing the G2-M block and the repair of the damage, thus counterbalancing the negative activity exerted by p53 upon damage at later time points. It would be interesting to investigate the possible modulation of the Chk1 promoter by TAp73 isoforms. Experiments are ongoing to elucidate this potential modulation. Recent experimental evidence suggests that a Chk1 dependent regulation of p73 occurs after DNA damage (Urist et al., 2004). Chk1 has been ascribed to have a role in the modulation of the stabilization and activation of E2F1 after DNA damage, which is involved in the activation of the TAp73 promoter, thus possibly attributing to Chk1 an indirect role in the apoptotic pathway induced by p73 activation.

The results obtained by the characterization of the 5' flanking region of the Chk1 gene support the evidence that the different players of the checkpoint pathways have to be considered as interconnected with each other in exerting their function.

The third part of my thesis (chapter 5) focuses on the investigation of the physiological role of Chk1 in cancer somatic cell lines. Much remains to be understood and clarified in terms of the role of this protein kinase in the cells, not only in the presence but also in the absence of genotoxic agents. The downregulation of Chk1 by siRNA in the HCT-116 isogenic cellular systems did not alter cell growth or compromise survival of these cells independently from the status of p53 and p21. This finding allowed the subsequent step of the investigation which addressed the effect of the lack of Chk1 in the presence of genotoxic agents. This observation together with the data recently obtained by Zachos et al. on the unique available vertebrate Chk1 KO somatic cellular system, led us to hypothesize that Chk1 probably was not essential for the survival of human somatic cell lines (Zachos et al., 2003). However the characterization of the mouse Chk1 deficient conditional KO in mammary glands model and the essential role attributed to Chk1 in mouse embryogenesis (not viability of Chk1 KO mice) (Takai et al., 2000; Lam et al., 2004), together with the data reported here on U2OS cells (chapter 5) strongly argue against the hypothesis that Chk1 is unessential for cell survival, a notion which can not be universally validated. In adult normal proliferating somatic cells *in vivo*, the lack of Chk1 resulted in cell lethality mediated by a currently unknown apoptotic pathway. Interestingly, Chk1 haploinsufficiency resulted in general cell cycle mis-coordination due to inappropriate entry into S phase, accumulation of DNA damage in S phase and uncontrolled mitotic entry in the presence of a damaged S phase (Lam et al., 2004). On

the other hand, the siRNA Chk1 transfected U2OS cells reported in this thesis provide an example of lethality observed in human somatic tumor cells at 72 hours post Chk1 downregulation, which occurs in part by apoptosis and in part by an unresolved mechanism (chapter 5.5). The effects of the lack of Chk1 in U2OS have been recently studied at 48 hours after Chk1 downregulation (Syljuasen et al., 2005). A general cell cycle mis-coordination similar to what has been described in the Chk1 heterozygotes mammary glands was observed, although no sign of apoptosis or altered cell morphology was found. One might hypothesize that the possible difference in extent of downregulation registered in this cell line between 48 and 72 hours post Chk1 siRNA transfection can in part explain the different result. The abnormal mitotic phenotype observed in HeLa cells at 72 hours, but not at 48 hours, post siRNA Chk1 transfection (chapters 5.2-5.4) could be explained by the hypothesis that cells must go through two cycles and a mitosis without Chk1 in order to cause lethality. From siRNA Chk1 downregulation in HeLa and U2OS cells and from the recent experimental evidences reported in the literature, the role played by Chk1 under unstressed conditions in regulating the normal progression of the cell cycle appears evident. This fact could correlate with the cell cycle dependent expression of Chk1 from S to G2-M phases, thus suggesting that this protein may have a more fundamental role in proliferating cells than in differentiated ones. It could be interesting to explore which genetic factor protects Chk1 deficient HCT-116 and lymphoma DT-40 cells from lethality. Nevertheless, to study the effect of Chk1 downregulation in a wider spectrum of human normal and cancer somatic cells and tissues could be a valuable strategy to characterize definitively the effects of the absence of Chk1. However the “complex world” represented by any

different normal and especially tumor cell type, might render this attempt very difficult, if not impossible. In any case, as death of nontumor proliferating cells limits the efficacy of many current chemotherapeutic treatments, if Chk1 inhibition causes toxicity, this may limit the effectiveness of the treatment. More efforts should be invested in the exploration of whether tumor cells gain resistance against the loss of Chk1 function more easily than normal cells, as this issue will be critical for the success of the inhibition of this protein as an anticancer strategy.

7.REFERENCES

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8.APPENDIX

8.1 LIST OF ABBREVIATIONS

| | |
|--------|-------------------------------------|
| 53BP1 | p53 binding protein 1 |
| 5-FU | 5-fluorouracil |
| aa | aminoacid |
| APC | anaphase promoting complex |
| ATM | ataxia-telangectasia mutated gene |
| ATP | adenosine 5' triphosphate |
| ATR | ATM-related gene |
| ATRIP | ATR-interacting protein |
| bp | base pairs |
| BRCA 1 | breast cancer-associated gene 1 |
| BSA | bovine serum albumin |
| CAK | cdk activating kinase |
| CDK | cyclin-dependent kinase |
| cDNA | complementary deoxyribonucleic acid |
| CFU | colony forming unit |
| Chk1 | checkpoint kinase 1 |
| Chk2 | checkpoint kinase 2 |
| CMV | cytomegalo virus |
| dCTP | deoxy-cytidine-5'-triphosphate |
| DDP | cis-dichloro-diamine-platinum |
| DMSO | dimethylsulphoxide |
| DNA | deoxyribonucleic acid |
| DNA-PK | DNA-dependent protein kinase |
| dNTP | nucleotides |
| DSB | double-strand breaks |
| DTT | dithiothreitol |

| | |
|------------------|--|
| ΔN | N-terminal deficient |
| ECL | enhanced chemiluminescence |
| EDTA | ethylenediaminetetraacetic acid |
| FCS | fetal calf serum |
| GST | Glutathione S-transferase |
| h | hour |
| H2AX | Histone-2A family, member X |
| HAT | Histone acetyl transferase |
| HEBS | HEPES-Buffered Saline |
| HU | Hydroxyurea |
| IC ₅₀ | concentration inhibiting the growth by 50% |
| IPTG | isopropyl β-D-thiogalactoside |
| IR | ionizing radiation |
| Kb | kilobase pairs |
| KDa | kilodaltons |
| KO | knock-out |
| l | liter |
| LB | Luria-Bertani |
| LOH | loss of heterozygosity |
| MCS | multiple cloning site |
| MDC1 | mediator of DNA damage checkpoint 1 |
| MDM2 | mouse double minute 2 |
| mRNA | messenger ribonucleic acid |
| NBS-1 | Nijmegen breakage syndrome-1 |
| OD | optical density |
| o/n | over night |
| PAC | P1 artificial chromosome |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PMSF | phenylmethylsulphonyl fluoride |

| | |
|------------|---------------------------------------|
| Rb | Retinoblastoma |
| RDS | radioresistant DNA synthesis |
| RNA | ribonucleic acid |
| RPA | replication protein A |
| SAM domain | sterile alpha-motif domain |
| SD | standard deviation |
| SDS | sodium dodecylsulphate |
| siRNA | small interfering RNA |
| SMC-1 | structural maintenance of chromosomes |
| ssDNA | single-strand DNA |
| TA | transactivation |
| TBS | tris buffered saline |
| TEMED | N',N,N',N'-tetramethylethylenediamine |
| Tet | Tetracycline |
| UV | ultra violet |
| UTR | untranslated region |
| V | Volts |
| wt | wild type |

8.2 LIST OF PUBLICATIONS

Full publications by the candidate on topics not associated with the work described here, and/or submitted before the commencement of this project:

Damia,G., Silvestri,S., Carrassa,L., Filiberti,L., Faircloth,G.T., Liberi,G., Foiani,M., and D'Incalci,M. (2001). Unique pattern of ET-743 activity in different cellular systems with defined deficiencies in DNA-repair pathways. *Int. J. Cancer* 92, 583-588.

Damia,G., Filiberti,L., Vikhanskaya,F., Carrassa,L., Taya,Y., D'Incalci,M., and Broggini,M. (2001). Cisplatin and taxol induce different patterns of p53 phosphorylation. *Neoplasia*. 3, 10-16.

D'Incalci,M., Erba,E., Damia,G., Galliera,E., Carrassa,L., Marchini,S., Mantovani,R., Tognon,G., Fruscio,R., Jimeno,J., and Faircloth,G.T. (2002). Unique features of the mode of action of ET-743. *Oncologist*. 7, 210-216.

Full publications by the candidate emanating from the work described in this thesis:

Carrassa,L., Broggini,M., Vikhanskaya,F., and Damia,G. (2003). Characterization of the 5'flanking region of the human Chk1 gene: identification of E2F1 functional sites. *Cell Cycle* 2, 604-609.

Carrassa,L., Broggini,M., Erba,E., and Damia,G. (2004). Chk1, but not Chk2, is involved in the cellular response to DNA damaging agents: differential activity in cells expressing or not p53. *Cell Cycle* 3, 1177-1181.

